In Search of New Genes Regulating the Development of Atherosclerosis

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DEDICATION

I dedicate this work to my parents for their love and support.

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1. Abbreviations

ApoE:	apolipoprotein E
BrdU:	5'-bromo-2'-deoxyuridine
HCD:	high cholesterol diet
HDC:	L-histidine decarboxylase
HDL:	high density lipoprotein
H1R:	histamine H1 receptor
H2R:	histamine H2 receptor
EC:	endothelial cell
E-sel:	E-selectin
IFNγ:	interferon gamma
IL:	interleukin
JNK:	c-jun N-terminal kinase
LDL:	low density lipoprotein
LDLr:	low density lipoprotein receptor
LOX-1:	lectin-like oxLDL receptor-1
L-sel:	L-selectin
MCP-1 (CCL2):	monocyte chemoattractant protein-1
mmLDL:	minimally modified low density lipoprotein
NFκB:	nuclear factor-kappa B
oxLDL	oxidized low-density lipoprotein
PDGF:	platelet-derived growth factor

PECAM-1 (CD31):	platelet/endothelial cell adhesion molecule-1
P-sel:	P-selectin
RANTES (CCL5):	regulated upon activation normal T-cell expressed and secreted (chemokine (C-C motif) ligand 5)
ROS	reactive oxygen species
SR-A:	scavenger receptor-A
TF:	tissue factor
Th:	T-helper
VCAM-1:	vascular cell adhesion molecule-1
VSMC:	vascular smooth muscle cell

2. Summary

Atherosclerosis is a progressive inflammatory disease, resulting from elevated blood cholesterol level and/or other risk factors such as hypercholesterolemia, arterial hypertension, diabetes mellitus, cigarette smoking, obesity, and aging. Endothelial cell (EC) dysfunction, arterial accumulation of modified lipoprotein particles, and activation of the immune system are important hallmarks of atherosclerosis. Development of atherosclerotic plaques leads to narrowing of blood vessels, reduced arterial blood flow and, as a result, life-threatening clinical complications like myocardial infarction or cerebral stroke.

In this study, we reveal several new mechanisms regulating atherosclerotic plaque formation using mouse models of atherosclerosis.

2.1 "Histamine H1 receptor promotes atherosclerotic lesion formation by increasing vascular permeability for low density lipoproteins"

Histamine (2-[4-imidazole]-ethylamine) is an endogenous amine, the product of L-histidine decarboxylation. Histamine is a well known mediator of inflammation and allergic responses. Most of the pro-inflammatory actions of histamine are mediated via the H1 receptor (H1R) and activation of nuclear factor-kappa B (NFκB). Interestingly, histamine was demonstrated to modulate several processes potentially important for the development of atherosclerosis.

In order to investigate the role of histamine signalling in atherogenesis, we used both a pharmacological and a genetic approach. *Apolipoprotein E* deficient

mice (*ApoE*$^{-/-}$), a widely used model of atherogenesis, were treated for 12 weeks with high cholesterol diet (HCD, Clinton-Cybulski diet, 1.25% cholesterol) and, in parallel, either with the H1R antagonist mepyramine or the H2R antagonist ranitidine. Moreover, we compared *ApoE / H1R* double mutants (*ApoE*$^{-/-}$ *H1R*$^{-/-}$) and *ApoE / H2R* double mutants (*ApoE*$^{-/-}$ *H2R*$^{-/-}$) to *ApoE*$^{-/-}$ mice after 12 weeks of HCD.

We observed that *ApoE*$^{-/-}$ animals treated with mepyramine, but not ranitidine, exhibited significantly reduced aortic plaque area compared with placebo-treated mice. Similarly, *ApoE*$^{-/-}$ mice genetically deficient in *H1R*, but not *H2R* displayed a 60% decrease in aortic plaque formation. Plasma cholesterol and triglyceride levels were not affected by the absence of *H1R*. In contrast, aortic permeability to ^{125}I-low density lipoproteins (^{125}I-LDL) was decreased in 18 week old *ApoE*$^{-/-}$ *H1R*$^{-/-}$ animals, both with and without a HCD. Consistent with this observation, bone marrow transplantation revealed that the presence of *H1R* on vascular cells determined plaque formation.

Lack of *H1R* resulted in decreased accumulation of lipids as well as macrophages and T-helper (Th) cells in atherosclerotic plaques. Moreover, reduced blood lymphocyte number and spleen size as well as decreased production of splenic Th1-specific cytokines were observed under these conditions. However, the reduced inflammation in *ApoE*$^{-/-}$ *H1R*$^{-/-}$ mice occurred secondary to alterations of the vessel wall, since bone marrow transplantation demonstrated that the development of atherosclerotic plaques did not depend on the presence or absence of H1R on bone marrow-derived cells.

Thus, the H1R promotes atherosclerosis by increasing arterial lipoprotein permeability, while the presence of the H1R on bone marrow-derived cells does not influence plaque formation.

2.2 "Accelerated early atherosclerosis in mice deficient in *L-selectin*"

L-selectin (L-sel) is a member of the selectin family of adhesion molecules. It is ubiquitously expressed in leukocytes and mediates the initial attachment of leukocytes to activated endothelium as well as lymphocyte homing to peripheral lymphoid tissues. Recently, it has been demonstrated that the leukocyte-endothelium interaction during atherogenesis is, at least partially, L-sel dependent.

To investigate the role of L-sel in atherosclerosis development *in vivo*, we generated double mutant *ApoE / L-sel* (*ApoE*$^{-/-}$ *L-sel*$^{-/-}$) mice. These animals, together with the corresponding *ApoE*$^{-/-}$ controls, were fed a HCD for a time period of 6 or 12 weeks. In addition, we compared 6 month old *ApoE*$^{-/-}$ to *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice without HCD.

We found that deletion of *L-sel* accelerated early, but not advanced, atherosclerosis. Plasma lipid profile was not affected by the absence of *L-sel*. The extent of leukocyte capture and rolling in atherosclerotic aorta *in vivo* did not depend on of the presence of *L-sel*. In line with this, the cellular composition of atherosclerotic plaque was similar in all groups of animals. There was also no difference in aortic expression of cytokines. The lack of *L-sel* affected leukocyte distribution in all monitored groups; the lymph nodes were significantly smaller

and exhibited reduced cellularity, whereas the spleens were larger as compared to *ApoE*$^{-/-}$ controls. Moreover, *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice exhibited increased blood leukocyte number.

Thus, L-sel protects from early, but not advanced, atherosclerosis; this effect does neither depend on plasma lipids nor leukocytes-blood vessel interactions.

2.3 "Endothelial overexpression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) increases LDL uptake and plaque formation"

ECs dysfunction is known to be a key process in early atherosclerosis. Increased permeability, expression of adhesion molecules, and endothelial-specific cytokines considerably accelerate the development of the disease. Lectin-like oxLDL receptor-1 (LOX-1) is postulated to be the main oxLDL scavenger receptor expressed by ECs. However, the role of LOX-1 in EC activation during atherogenesis is not well understood.

To address this issue, we have generated transgenic mice overexpressing LOX-1 specifically in ECs. We fed these animals and the corresponding wild-type controls with a HCD for 30 weeks to investigate the role of LOX-1 in early atherosclerosis. We also crossbred *LOX-1* transgenic (*LOX-1 TG*) animals with *ApoE*$^{-/-}$ mice to monitor the role of endothelial-specific LOX-1 overexpression in more advance stages of the disease.

LOX-1 overexpression in ECs resulted in accelerated atherogenesis in both wild-type and *ApoE*$^{-/-}$ mice, which was associated with impaired EC-

dependent relaxation to acetylcholine. Further, *LOX-1 TG* mice exhibited increased expression of adhesion molecules and enhanced accumulation of leukocytes in atherosclerotic plaques. Plasma lipids were similar in all groups of animals.

These results indicate that the expression of LOX-1 by ECs is an important factor promoting atherogenesis.

2a. Deutsche Zusammenfassung der Doktorarbeit

Die Arteriosklerose ist eine chronische inflammatorische Erkrankung, welche oftmals aus einem erhöhten Cholesterin Plasma Level resultiert. Die zur Entwicklung dieser Erkrankung beitragenden Risikofaktoren beinhalten unter anderem Hypercholesterinämie, Hypertonie, Diabetes, Rauchen, Übergewicht und Alter. Kennzeichen der Arteriosklerose sind Endothelzellen (EC)-Dysfunktion, arterielle Anhäufungen von modifizierten Lipoprotein-Partikeln und Aktivierung des Immunsystems. Die Entwicklung von arteriosklerotischen Plaques führt zu einer Verengung der Blutgefässe, einem reduzierten arteriellen Blutfluss und lebensbedrohenden klinischen Folgeschäden wie beispielsweise Herzinfarkt oder Schlaganfall.

In dieser Studie zeigen wir mittels Arteriosklerose-Mausmodellen mehrere neue Mechanismen, die bei der Entstehung von arteriosklerotischen Plaques bedeutsam sind, auf.

2.1a "Histamin H1 Rezeptor fördert die arteriosklerotische Plaque Bildung durch erhöhte vaskuläre Permeabilität für Lipoproteine niederer Dichte"

Histamin (2-[4-Imidazol]-Ethylamin) ist ein endogenes Amin und das Produkt der L-Histidin Decarboxylierung. Des Weiteren ist Histamin ein bekannter Mediator von Entzündungs- und Allergiereaktionen. Die meisten der proinflammatorischen Wirkungen von Histamin werden über den H1 Rezeptor (H1R) und die Aktivierung des Nukleären Faktors κB (NFκB) vermittelt.

Interessanterweise wurde gezeigt, dass Histamin mehrere potentiell für die Entwicklung der Arteriosklerose wichtige Prozesse reguliert.

Um die Rolle der Histamin-vermittelten Signalwirkung in der Arteriosklerose zu untersuchen, haben wir sowohl einen pharmakologischen wie auch einen genetischen Ansatz angewendet. *Apolipoprotein E* Knock-out Mäuse (*ApoE*$^{-/-}$), ein bekanntes Modell der Arteriosklerose, wurden mit einer 12-wöchigen Hoch-Cholesterin Diät (HCD, Clinton-Cybulski Diät, 1.25% Cholesterin) und parallel dazu entweder mit dem H1R Antagonisten Mepyramin oder dem H2 Rezeptor (H2R) Antagonisten Ranitidin behandelt. Des Weiteren wurden *ApoE / H1R* Doppel-Knock-out Mäuse (*ApoE*$^{-/-}$ *H1R*$^{-/-}$) und *ApoE / H2R* Doppel-Knock-out Mäuse *(ApoE*$^{-/-}$ *H2R*$^{-/-}$) mit *ApoE*$^{-/-}$ Mäusen nach der 12-wöchigen HCD verglichen.

Wir haben beobachtet, dass *ApoE*$^{-/-}$ Tiere, die mit Mepyramin behandelt wurden, signifikant weniger arteriosklerotische Plaques als die mit Placebo oder Ranitidin behandelten Mäuse aufwiesen. Zudem resultierte die genetische *H1R*, nicht aber *H2R*, Defizienz in *ApoE*$^{-/-}$ Mäusen in einer um 60% verminderten Atherom Entstehung. Der Lipidgehalt im Plasma wurde durch das Fehlen des *H1R* dagegen nicht beeinträchtigt. Im Gegensatz dazu war die Durchlässigkeit der Gefässwand für ^{125}I-LDL in 18-wöchigen *ApoE*$^{-/-}$ *H1R*$^{-/-}$ Tieren vermindert, sowohl in solchen mit als auch in solchen ohne HCD. Übereinstimmend mit diesen Beobachtungen deckte eine Knochenmarktransplantation auf, dass die Präsenz des H1R auf Zellen der Gefässwand für die Ausbildung von arteriosklerotischen Plaques bedeutsam ist.

Das Fehlen des *H1R* resultierte in reduzierter Akkumulation von Lipiden und auch Makrophagen sowieT-Helfer Zellen (Th) in den arteriosklerotischen Plaques. Des Weiteren wurde in der Abwesenheit des *H1R* eine Verminderung der Lymphozyten im Blut, der Milzgrösse sowie der Produktion der Th1-spezifischen Cytokine beobachtet. Die geringere Endzündungsreaktion in *ApoE*$^{-/-}$ *H1R*$^{-/-}$ Mäusen trat sekundär zur Änderung der Gefässwand auf, weil die Knochenmarktransplantation aufzeigte, dass die Entwicklung von arteriosklerotischen Plaques nicht von der Anwesenheit oder Abwesenheit des *H1R* auf Zellen, die vom Knochenmark stammen, abhängt.

Wir kamen zum Schluss, dass der *H1R* die Entwicklung von arteriosklerotischen Plaques über eine erhöhte Durchlässigkeit der Blutgefässe für LDL fördert, während die Anwesenheit des H1R auf Zellen, die vom Knochenmark stammen, die Entwicklung von arteriosklerotischen Plaques nicht beeinflusst.

2.2a "Beschleunigte frühe Arteriosklerose in *L-Selektin* Knock-out Mäusen"

L-Selektin (L-sel) ist ein Mitglied der Familie der Selektin Adhäsions-Moleküle. Es wird ubiquitär in Leukozyten exprimiert und vermittelt sowohl die anfängliche Anhaftung von Leukozyten zur Aktivierung des Endothels als auch das Lymphozyten-Homing zum periphereren Lymph-Gewebe. Kürzlich wurde gezeigt, dass die Interaktion zwischen Leukozyten und Endothel während der Entstehung der Arteriosklerose, zumindest teilweise, L-sel abhängig ist.

Um die Rolle von L-sel in der Entstehung der Arteriosklerose *in vivo* zu untersuchen, haben wir *ApoE / L-sel* Doppel-Knock-out Mäuse (*ApoE*$^{-/-}$ *L-sel*$^{-/-}$) generiert. Diese Tiere, zusammen mit den entsprechenden *ApoE*$^{-/-}$ Kontrollen, wurden während 6 oder 12 Wochen einer HCD unterzogen. Zusätzlich haben wir halbjährige *ApoE*$^{-/-}$ Mäuse mit gleich alten *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ Tieren, die nicht einer HCD unterzogen wurden, verglichen.

Wir haben herausgefunden, dass die genetische Defizienz von *L-sel* die frühe, jedoch nicht die späte Arteriosklerose beschleunigt. Der Lipidgehalt im Plasma wurde durch das Fehlen von *L-sel* nicht beeinträchtigt. Zudem war das Ausmass des Leukozyten Einfangens und Rollens in der arteriosklerotischen Aorta *in vivo* unabhängig von der Präsenz von *L-sel.* Übereinstimmend mit diesen Beobachtungen wurde gezeigt, dass die zelluläre Zusammensetzung der arteriosklerotischen Plaques in sämtlichen Tiergruppen ähnlich war. Es gab auch keinen Unterschied in der Expression von Cytokinen in der Aorta. Die Absenz von *L-sel* beeinflusste wie erwartet die Leukozyten Verteilung in allen beobachteten Gruppen. Zudem waren die Lymphknoten signifikant kleiner und wiesen eine reduzierte Zellularität auf und die Milz war grösser als diejenige der *ApoE*$^{-/-}$ Kontrollen. Des Weiteren wiesen *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ Mäuse eine erhöhte Anzahl von Leukozyten im Blut auf.

Demzufolge schützt L-sel vor der frühen, aber nicht vor der späten Arteriosklerose. Dieser Effekt hängt weder von den Lipiden im Plasma noch von der Leukozyten-Blutgefäss Interkation ab.

2.3a "Die Überexpression von LOX-1 im Endothel erhöht die Aufnahme von LDL und die Plaque Bildung"

Als ein entscheidender Prozess in der frühen Arteriosklerose hat sich die EC-Dysfunktion erwiesen. Erhöhte Durchlässigkeit, Expression von Adhesionsmolekülen und Endothel-spezifische Cytokine beschleunigen die Krankheitsentwicklung beträchtlich. LOX-1 gilt als der hauptsächliche oxLDL Scavenger Rezeptor, der von EC exprimiert wird. Jedoch ist die Rolle von LOX-1 in der Aktivierung der EC und der Entwicklung der Arteriosklerose noch nicht vollständig bekannt.

Um diese Frage anzugehen, haben wir transgene Mäuse generiert, die LOX-1 spezifisch in EC überexprimieren. Wir haben diese Tiere sowie die entsprechenden Wildtypen einer 30-wöchigen HCD unterzogen, um die Rolle von LOX-1 in der frühen Arteriosklerose zu untersuchen. Zudem haben wir *LOX-1* transgene *(LOX-1 TG)* Tiere mit *ApoE*$^{-/-}$ Mäusen gekreuzt, um die Endothel-spezifische LOX-1 Überexpression während späteren Stadien der Krankheit zu studieren.

Die Überexpression von LOX-1 in EC resultierte in einer beschleunigten Arteriosklerose Entwicklung, sowohl in Wildtypen als auch in *ApoE*$^{-/-}$ Mäusen, was einer verminderten EC-abhängigen Relaxation auf Acetylcholin nahesteht. Zudem zeigten *LOX-1 TG* Mäuse eine erhöhte Expression von Adhesionsmolekülen sowie eine Anhäufung von Leukozyten in arteriosklerotischen Plaques. Der Lipidgehalt im Plasma war ähnlich in allen Tiergruppen.

Unsere Resultate deuten darauf hin, dass die Expression von LOX-1 in EC ein wichtiger Faktor der Arteriosklerose Entwicklung ist.

3. Introduction

Complications of atherosclerosis are the leading cause of death in Western societies (Fig. 1). Atherosclerosis is a progressive inflammatory disease characterized by accumulation of cells, lipids, and extracellular matrix in the vessel wall eventually leading to narrowing of vessels and clinical manifestations like myocardial infarction or cerebral stroke. Endothelial dysfunction, LDL oxidation, and leukocyte accumulation are key events in atherosclerosis [1-3]. Although our knowledge of the pathophysiology of this disease has improved substantially over the last years, molecular events occurring in the pathogenesis of atherogenesis are still poorly understood.

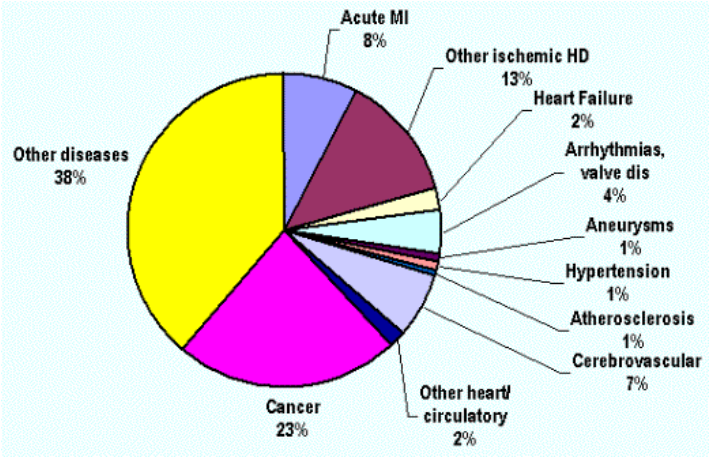

Figure 1

Complications of atherosclerosis are the main cause of death in developed countries; adapted from National Vital Statistics Reports, USA, 2003.

3.1 Risk factors

Epidemiological studies reveal that complications of atherosclerosis are responsible for the majority of deaths in developed countries.

Endothelial dysfunction is an early event in atherogenesis. The healthy endothelium helps to maintain integrity of blood vessel, regulates vasomotion, and actively inhibits thrombosis and inflammation. Thus, factors that injure ECs contribute to the development of the disease. Several risk factors, such as hypercholesterolemia or homocysteinemia, have been identified for causing endothelial dysfunction.

Typical Western diet, rich in triglycerides and cholesterol, is undoubtedly one of the reasons of increased prevalence of the disease [1]. Indeed, a large body of evidence points to hypercholesterolemia as one of the most serious risk factors for the development of atherosclerosis [4]. It has been demonstrated that an increased risk of coronary artery disease correlates with elevated plasma cholesterol levels. Especially dangerous are high LDL plasma levels, the major transporter of cholesterol in the blood stream [5, 6]. Accumulation of LDL in the arterial intima followed by subsequent oxidation and acetylation are key events in formation of atherosclerotic plaques, a hallmark of atherosclerosis [1].

Also individuals suffering from hypertension are more prone to development of atherosclerosis, coronary artery disease, and stroke [6]. Other factors associated with endothelial dysfunction predisposing to atherosclerosis are diabetes, aging, and cigarette smoking [6-9].

3.2 Pathogenesis of atherosclerosis

3.2.1 Fatty streak formation

The intima, composed of a single layer of endothelium and underlying connective tissue, maintains integrity of the blood vessel [10]. Early atherosclerosis is characterized by the accumulation of LDL in the arterial intima, occurring secondary to activation of EC and increased permeability of the vessel wall. Factors modulating vascular permeability, such as histamine, have been implicated in atherosclerosis, but their *in vivo* studies relevance is unclear [11, 12].

Hypercholesterolemia is a major risk factor predisposing to fatty streak formation [4]. Indeed, the stimulation of endothelial LOX-1 i*n vitro* with LDL results in cell activation [13]. It remains to by clarified, whether this process affects atherosclerosis development *in vivo*.

LDL accumulated in the vessel wall become eventually modified by reactive oxygen species (ROS) and 15-lipoxygenase or other enzymes first to minimally modified LDL (mmLDL) and then to oxidized LDL (oxLDL) [14]. mmLDL is a potent chemoattractant molecule, which further accelerates formation of atherosclerotic changes. Accumulation of oxLDL in the vessel wall accounts for the formation of the earliest morphological manifestation of atherosclerosis - the fatty streak [1, 15].

Some of the main risk factors associated with atherosclerosis like hypertension, diabetes or cigarette smoking exert their negative effects primarily via activation of the endothelium [7, 8, 16].

3.2.2 Attraction of leukocytes

Under normal conditions, ECs resist prolonged contact with blood leukocytes. However, when activated, both ECs and leukocytes start to express adhesion molecules thus facilitating attachment and migration of cells into sites of inflammation [10]. The recruitment of leukocytes is regulated by different families of adhesion molecules. Expression of particular adhesion molecules and their interaction with ligands regulates the specificity of leukocyte migration into the arterial wall [1, 17]. The initial phase of an inflammatory response is characterized by the slowing down of leukocytes, which allows their firm adhesion and transmigration. The molecules responsible for initial rolling and tethering of leukocytes are called selectins [17, 18]. The selectin family of adhesion molecules consists of E-selectin (E-sel), P-selectin (P-sel), and L-sel. E-sel and P-sel mediate the attachment of leukocytes to the endothelial layer before firm adhesion mediated via integrins, vascular cell adhesion molecule-1 (VCAM-1), and platelet/endothelial cell adhesion molecule (PECAM-1) occurs (Fig. 2) [17, 18]. The importance of E-sel and P-sel in atherosclerosis *in vivo* was demonstrated with genetically modified mice; in contrast the role of L-sel remains to be clarified. In addition, expression of several chemoattractant proteins enhances accumulation of leukocytes in atherosclerotic plaques. Mouse models

show that the presence of certain molecules like chemokine (C-C motif) ligand 5 (CCL5), interferon gamma (IFNγ), monocyte chemoattractant protein-1 (MCP-1), and IL-8 is important for leukocyte transmigration into sites of inflammation [1].

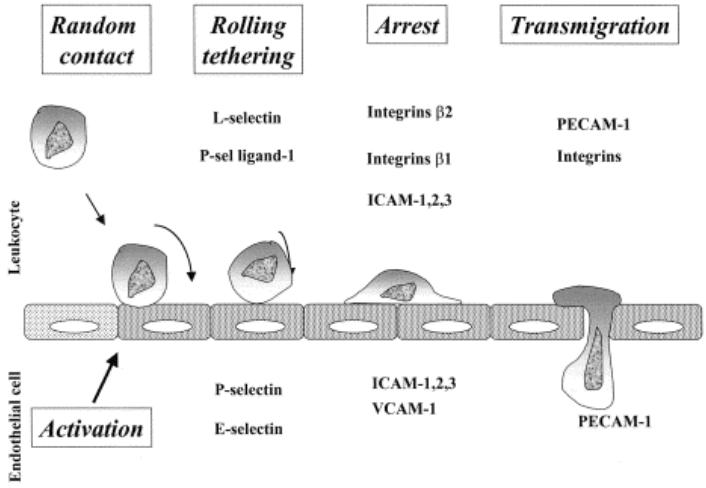

Figure 2

Rolling and tethering of leukocytes along activated endothelium is mediated primarily via the selectin family of adhesion molecules. Arrestment and transmigration occur due to interactions of integrins with their ligands expressed by ECs; adapted from Blankenberg et al [17].

3.2.3 Formation of an atherosclerotic plaque

Macrophages express several scavenger receptors specific for modified LDL such as scavenger receptor A (SR-A) or CD36. Although these two together mediate the uptake of the majority of cholesterol, their role in atherosclerosis development remains controversial [19, 20].

Once transmigrated to the arterial intima, macrophages start to scavenge oxLDL particles and undergo morphological changes to become **foam cells**, the

hallmark of early atherosclerotic lesions. Foam cells express several proinflammatory molecules like tissue factor (TF), platelet-derived growth factor (PDGF), several cytokines, metalloproteinases, and produce ROS, all of which contribute to attract other leukocytes, stimulate vascular smooth muscle cell (VSMC) migration, and exacerbate inflammation [1].

Following transmigration of monocytes, a subsequent key step in atherogenesis is accumulation of lymphocytes. T cells are relatively low in number, but represent a very important component of the atherosclerotic plaque [21]. Several animal studies emphasize the importance of lymphocytes, in particular Th $CD4^+$ cells in atherogenesis [2, 21, 22]. Indeed, deficiency of adaptive immunity leads to reduced atherosclerosis in mice lacking *ApoE*, whereas reconstitution of immuno-deficient mice with $CD4^+$ T cells accelerates atherogenesis [21, 22].

Both activated endothelium and lipid loaded foam cells express PDGF, a potent attractant of and mitogen for VSMCs. Progression of atherosclerotic lesion formation is associated with migration of VSMCs from the media to the activated intima. VSMCs not only secrete large amounts of connective tissue into the plaque, but can also scavenge modified LDL particles and become foam cells [23] (Fig. 3).

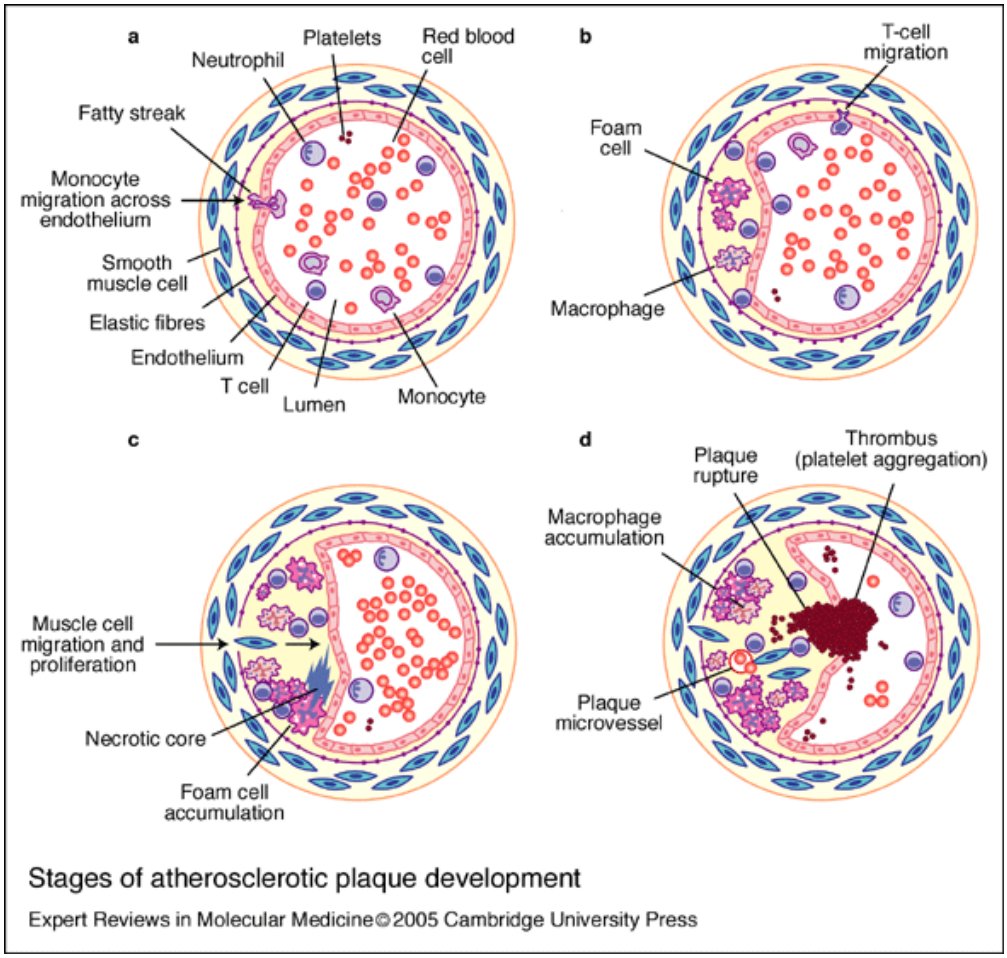

Figure 3

Formation of advanced atherosclerotic plaque; adapted from Stevens et al [24].

3.2.4 Complications of atherosclerosis

Formation of fibrous plaques with a central necrotic core, which may eventually lead to clinical complications, is a typical feature of advanced atherosclerosis. Unlike fatty streaks, an advanced atherosclerotic plaque can be easily visualized macroscopically. It is composed of a fibrous cap containing extracellular connective tissue matrix and VSMCs as well as lipid core containing mainly foam cells, cholesterol crystals, and cellular debris [1]. Progression of

plaque development is associated with narrowing of the vessel lumen (stenosis) and reduced blood flow. This can result in ischemic injury of the organ supplied by the diseased vessel [25].

Thickness and composition of the fibrous plaque are critical determinants of plaque stability. Substantial lipid core and thin fibrous cap are typical features of unstable plaques. In contrast, thick fibrous cap and relatively small lipid core are more characteristic of stable plaques. Rupture of unstable plaques is a very common complication of atherosclerosis. It results in exposure of TF-rich plaque components to the blood stream and leads to thrombosis and eventually vessel occlusion [26] (Figure 3). Rupture of atherosclerotic plaques may have different clinical manifestations depending on the vessel localization, for example stroke if plaque rupture occurs in the vessel supplying the brain, or myocardial infarction if plaque rupture occurs in the coronary circulation. In addition, fragments of disrupted atherosclerotic lesions may embolize to distal sites [25].

Development of late stages of atherosclerosis is associated with weakening of the vessel wall and loss of elastic tissue leading to localized, blood-filled dilations called aneurysms. Aneurysm formation affects most often the abdominal portion of the aorta, the blood vessels supplying the brain (circle of Willis), and other large arteries. Rupture of the aneurysm leads to internal bleeding and is a serious life threatening condition [27].

3.3 Role of histamine in atherogenesis

Rozenberg, I., Tanner, FC, Luescher, TF. 2008. Histamine. In *Cardiovascular hormone systems, from molecular mechanism to novel therapeutics*. M. Bader, editor. Weinheim: Wiley-Blackwell. 295-314.

Cardiovascular Hormone Systems

Histamine

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1. Introduction

Due to its numerous effects and possible therapeutic potential, histamine is one of the most intensely studied mediators in medicine. Histamine is an endogenous amine produced in a one step reaction from L-histidine. It regulates vasoreactivity, coagulation as well as immunological responses. An increased production of histamine has been noted during allergic action, within atherosclerotic plaques, in patients with stable coronary artery disease, and in patients with acute coronary syndromes [1-3]]. Most of the histamine is released by activated mast cells and basophils, but also by macrophages, lymphocytes, platelets, and other cells [3-8]. Mast cells store histamine in secretory vesicles and only release it upon stimulation with specific ligands (i.e. IgE) activating surface receptors. Degranulation of mast cells causes fast, but transient increases in histamine concentration, resulting in hypersensivity, vascular permeability, vasodilation, or vasoconstriction [9].

Recently, it has been demonstrated that the histamine-producing enzyme, L-histidine decarboxylase (HDC), is expressed in activated macrophages from human atherosclerotic plaques. Since macrophages, unlike mast cells, do not contain secretory vesicles, macrophage-derived histamine is not stored, but rather produced *de novo* [10, 11]. Therefore, macrophage stimulation does not cause sudden increases in local histamine concentration, but rather provides persistently increased histamine tissue levels.

The diverse effects of histamine are due to differential expression and regulation of the four histamine receptors (see Section 3).

2. Biochemistry

2.1 Synthesis:

Histamine (2-[4-imidazole]-ethylamine) is an endogenous amine, the product of L-histidine decarboxylation. Its synthesis is controlled primarily by the availability of L-histidine and the activity of HDC [12], the rate limiting enzyme controlling histamine biosynthesis (Figure 2.1.1). Activity of HDC is regulated mainly at the transcriptional level, at least in cultured enterochromaffin-like cells [13, 14]. The enzyme has been detected in various cells of the immune system, gastrointestinal and bronchial endocrine cells, neuroendocrine cells, as well as some tumor cells [15]. Histamine may be released in response to various immunological (immunoglobulin E, cytokines) and non-immunological (compound 48/80, calcium ionophore, opioids) stimuli.

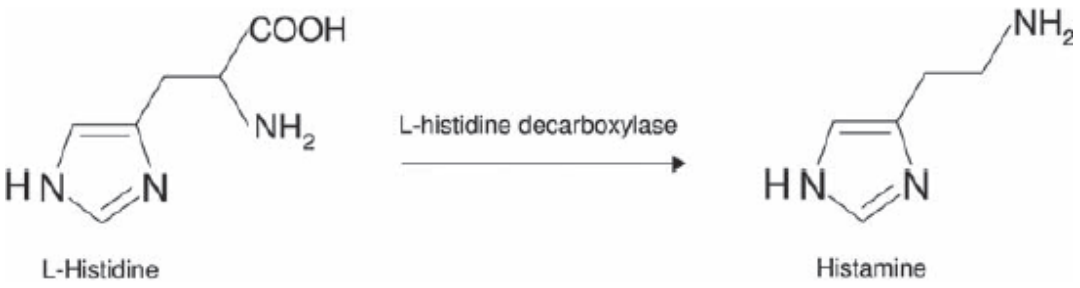

Figure 2.1.1 Biosynthesis of histamine from L-histidine.

2.2 Degradation:

In order to avoid excessive inflammatory reactions, histamine's action has to be highly regulated by limited production but also degradation. Histamine inactivation occurs either through metabolization into inactive compounds and/or cellular uptake. Indeed, many cells express high affinity receptors specific for monoamines like histamine. One recently characterized receptor is the organic cation transporter (OCT)-2. On the other hand, histamine is inactivated is by two catabolic pathways: (i) methylation by histamine N-methyltransferase (HNMT) and (ii) oxidative deamination by diamine oxidase. Since histamine cannot easily enter cells and HNMT seems to be localized primarily in the cytoplasm, there are two hypotheses of histamine metabolization: the transporter hypothesis and the membrane hypothesis. The transporter hypothesis assumes that histamine is transported via special histamine transporters into the intracellular space, where it is metabolized by HNMT. According to the second hypothesis, HNMT upon stimulation is translocated to the plasma membrane, where it has easy access to histamine [16].

3. Receptors

The pleiotropic and tissue-specific effects of histamine are mediated via four G-coupled protein membrane receptors (histamine H1-H4 receptors; Table 3.1). Effects of histamine upon histamine receptor ligation are very complex and often contradictory; depending on the pattern of receptors it activates [17].

Table 3.1 Expression and function of histamine receptors.

Type of receptor	**Expression**	**Molecular pathway involved**	**Function**
Histamine H1 receptor	EC, SMC, leukocytes, nerve cells	phospholipase C via $G_{q/11}$ family of G proteins	allergy and inflammation
Histamine H2 receptor	EC, VSMC, leukocytes, gastric parietal cells, nerve cells	couple to adelylyl cyclase via GTP-binding protein G_s	gastric acid secretion, inflammation
Histamine H3 receptor	neurons, leukocytes	inhibition of cAMP via $G_{i/o}$	neurotransmission
Histamine H4 receptor	leukocytes, nerve cells	inhibition of cAMP via $G_{i/os}$	allergy and inflammation

Histamine H1 receptor is coupled to the $G_{q/11}$ protein and the phosphoinositol hydrolysis pathway resulting in enhancement of inflammatory processes. Its activation induces signs of allergic reactions, such as bronchoconstriction, increased vascular permeability, and vasodilation due to nitric oxide (NO) release from

vascular endothelial cells (ECs). Mice lacking the histamine H1 receptor develop normally, but exhibit behavioral changes, such as reduced exploratory behavior and altered circadian rhythm as well as increased daily food intake [18]. These observations confirm the importance of histamine HI receptor signaling in the brain. Histamine H1 receptor deficiency also attenuates airway allergic inflammation, which is associated with decreased levels of T-helper 2 cells (Th2), cytokine production, and circulating IgE in bronchoalveolar lavage fluid [19].

The histamine H2 receptor is coupled to the $G\alpha_s$ protein and cAMP signaling. Acting via the H2 receptor, histamine regulates inflammatory responses, angiogenesis, and gastric acid secretion. Homozygous mutant mice lacking histamine H2 receptor are viable and, surprisingly, exhibit normal basal gastric pH. This observation is clearly distinct from the elevated gastric pH observed in mice treated with H2 receptor antagonists [20].

The histamine H3 receptor has been detected in the central as well as peripheral nervous system and negatively regulates the release of histamine and other neurotransmitters as well. Mice treated with histamine H3 receptor antagonists exhibit behavioral changes, such as enhanced spatial learning and reduced anxiety. A study with H3 receptor knockout animals as well as with H3 receptor agonist treated animals demonstrated a role of H3 receptor in energy homeostasis and suggested a therapeutic potential for H3 receptor ligands in the treatment of obesity and diabetes mellitus [21]. The recently discovered histamine H4 receptor involves $G_{i/os}$ protein signaling and enhances intracellular Ca^{2+} levels. Activation of histamine H4 receptor stimulates accumulation of inflammatory cells, primarily eosinophils and

mast cells, and activates proinflammatory responses. H4 receptor-deficient mice as well as mice treated with H4 receptor antagonist exhibit diminished allergic pulmonary inflammation, decreased infiltration of lung eosinophils and lymphocytes, and decreased Th2 responses [22].

4. Vasomotion

The role of histamine in the regulation of vasomotion is complex and varies depending on its concentration and the receptor it activates. Both the contracting and the relaxing responses in bronchial arteries are significantly inhibited by mepyramine, suggesting that the H1-receptor is involved [23]. In contrast, another study demonstrated that H2 receptor activation is crucial for NO production and vascular relaxation in human arteries and veins [24]. Hence, the involvement of histamine receptors in vasomotion regulation is complex. Evidence indicates that the vasodilator response is related to the release of NO from ECs, and that the vasoconstrictor effect is resulting from a direct action of histamine on vascular smooth muscle cells (VSMCs). Indeed, histamine-dependent arterial dilation, but not constriction, is blunted in mice lacking endothelial nitric oxide synthase (eNOS, also classified as NOSIII) [25]. However, contraction of VSMCs cells triggered by histamine can be at least in part endothelium-dependent due to the action of endothelium-derived contracting factor (EDCF). It has been proposed that stimulation with low concentrations of histamine results in relaxation of human vessels, whereas higher concentrations exert an opposite effect [26]. Importantly, the action of histamine differs depending on the vascular bed involved. In mammary artery, it induces endothelium-dependent relaxation, whereas it promotes contraction in saphenous vein (Figure 4.1) [27].

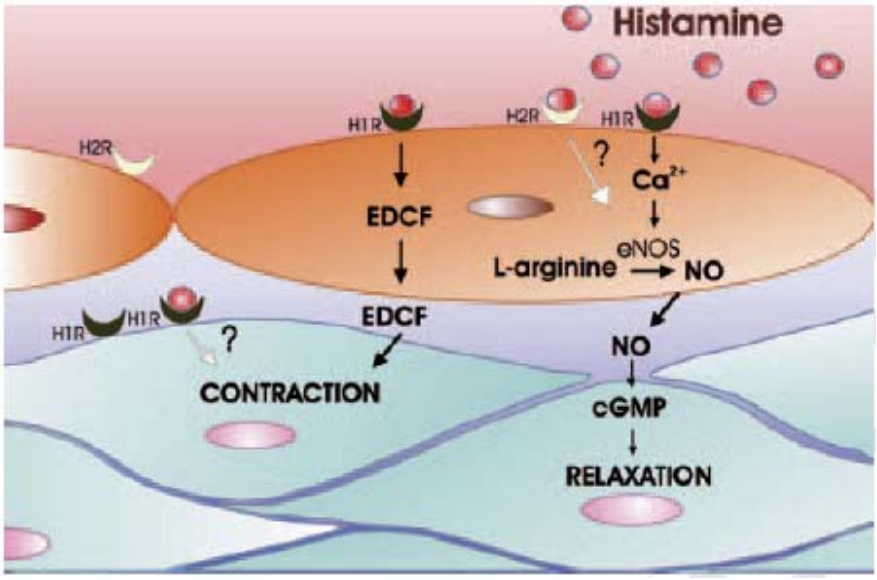

Figure 4.1 Dual role of histamine in regulation of vascular tone. Histamine acting via histamine H1 receptor (H1R) increases eNOS activity and enhances NO production in ECs through increasing intracellular Ca^{2+} levels. Release of NO from EC causes relaxation of VSMCs. Concomitantly, histamine-triggered release of EDCF from ECs may cause contraction of VSMCs.

4.1 EDRF

In the past, VSMCs were considered to be the sole regulator of vascular tone. VSMCs, the most abundant component of the vessel wall, are indeed known to contain large numbers of contractile proteins, determining the resistance of vessels and therefore regulating blood flow. However, in 1980, Furchgott and Zawadzki observed that anatomical integrity of the EC layer is crucial for acetylcholine-mediated relaxation of VSMCs [28]. This observation proved the importance of the endothelium in the vasoconstriction-vasodilatation balance. The compound discovered in 1980 was initially, based on its function, named endothelium-derived relaxing factor (EDRF) and later characterized as NO [29]. Since this discovery,

various endothelium-derived vasodilators (prostacyclin) and vasoconstrictors (endothelin-1, angiotensin II, prostaglandins) have been identified as well. Together with NO, these compounds contribute to the regulation of vascular tone [30]. Histamine plays a role in endothelial NO release, thereby promoting vasodilation. NO dilates blood vessels by stimulating soluble guanylyl cyclase and increasing cGMP in VSMCs [31, 32]. NO is synthesized from L-arginine by at least three distinct NOS isoforms. Production of NO in ECs is regulated by the constitutively expressed eNOS. In response to stimuli such as shear stress, acetylcholine, or histamine, eNOS activity and NO production in ECs increases.

In addition to its vasoactive properties, NO also prevents platelet aggregation and leukocyte adhesion as well as VSMC proliferation, which may be relevant in the development of cardiovascular diseases like atherosclerosis [33-35]. The importance of NO release by ECs in blood pressure regulation has been confirmed with eNOS deficient animals, which develop hypertension [36]. It has also been reported, that NO production is diminished in patients with elevated blood pressure when compared to normotensive individuals [37].

4.1.1 Signaling Role of Ca^{2+}

Production of NO is regulated primarily by modulation of eNOS activity in a Ca^{2+}-and calmodulin-dependent manner. After synthesis eNOS is translocated to specialized signal-transducing plasmalemmal compartments called caveolae. Caveolin, the main structural component of caveolae, interacts with eNOS leading to enzyme inhibition. It has been demonstrated in human ECs that the inhibitory action of calveolin on

eNOS activity is modulated by Ca^{2+}/calmodulin. Addition of calmodulin indeed disrupts the eNOS-calveolin complex in a Ca^{2+} dependent manner [38].

H1 receptor stimulation results in activation of phospholipase C and the production of inositol-1,4,5-triphosphate from phosphatidylinositol (4,5)-bisphosphate, which triggers the release of Ca^{2+}. Accordingly, histamine increases eNOS activity and enhances NO production at least in part through increase of intracellular Ca^{2+} [39].

4.1.2 Activation of eNOS

Phosphorylation represents a crucial post-translational regulatory mechanism of enzyme activation. In case of eNOS, phosphorylation on serine or tyrosine residues is probably involved in intracellular enzyme translocation followed by its activation. Interestingly, histamine-mediates eNOS phosphorylation at serine 1177, independent of Ca^{2+} signaling. It has been shown, that inhibition of protein kinase A as well as 5'-AMP-activated protein kinase (AMPK) strongly inhibits phosphorylation and activation of eNOS triggered by histamine stimulation [40].

4.1.3 Transcriptional Regulation of eNOS

Regulation of gene expression is a very common way to modulate biological pathways. Recently, it has been shown that histamine does not only play a role in activation of eNOS, but also affects the expression level of the enzyme. Indeed, histamine upregulates gene expression of endothelial nitric oxide synthase in cultured human ECs in a Ca^{2+}-dependent manner. The enhanced expression of

eNOS could be prevented by mepyramine, a selective antagonist of histamine H1 receptor, but not by histamine H2 and histamine H3 receptor antagonists [41].

4.2 EDCF

ECs can not only induce relaxation, but also contraction of underlying VSMCs. The molecule responsible for endothelium-dependent contraction was initially called EDCF. Indeed, various mediators produced by the endothelium such as endothelin-1 or thromboxane A2 were later found to induce vascular constriction. It has been demonstrated that histamine may trigger either constriction or relaxation depending on its concentration and the animal model used. The histamine-mediated constriction of VSMCs was blocked by histamine H1 receptor inhibitors, but increased after application of histamine H2 receptor inhibitors in a canine model [42]. Similarly, histamine induced strong constriction in rabbit basilar artery [43]. These results suggest that histamine, activating the H1 receptor, plays a role in endothelium-derived contraction, presumably via the generation of EDCF, which can be modulated by the concomitant release of endothelium-derived NO via activation of the H2 receptor.

5. Thrombosis

Coronary atherosclerosis complicated by plaque rupture and thrombosis is primarily responsible for the development of acute coronary syndromes. Under normal conditions, however, the endothelial layer provides a barrier between the blood and the vessel wall; it maintains blood fluidity and is crucial for regulating the interaction between blood and solid tissue components. Hence, ECs are able to prevent procoagulant and thrombotic events. They synthesize negatively charged heparin sulfate proteoglycans (glycocalix), maintaining the vascular wall in an antithrombotic state by accelerating the inhibition of factor Xa and thrombin via antithrombin III. ECs are also the main source of tissue factor pathway inhibitor (TFPI). Binding of TFPI to activated factor Xa leads to the formation of an inactive tissue factor-VIIa-Xa complex. Furthermore, the endothelium expresses the receptor for thrombomodulin, which modifies thrombin specificity and accelerates thrombin mediated conversion of precursor protein C to activated protein C. Finally, the sequestration of two prothrombotic molecules - tissue factor as well as von Willebrand factor (vWF) accounts for the antithrombotic properties of ECs. Under certain conditions like inflammatory stimulation, however, ECs participate in activation of coagulation. Mast cells, the main source of histamine, tend to redistribute and accumulate at sites of thrombosis. Histamine indeed promotes a series of prothrombotic events like activation of ECs, induction of tissue factor expression, and stimulation of vWF release. Furthermore, histamine is released by platelets during thrombotic events, and exogenous histamine dose-dependently enhances platelet aggregation induced

by various stimuli. Conversely, it has been demonstrated that mast cell-deficient mice are hyperresponsive to thrombogenic stimuli, probably due to lack of mast cell-derived heparin [44].

5.1 Tissue Factor Expression

Tissue factor is a 263-residue membrane bound glycoprotein, member of the type II cytokine receptor family, controlling initiation of the extrinsic coagulation cascade, since it is the cellular receptor for activated factor VII. The endothelial layer provides a barrier between the blood and the tissue factor-rich vessel wall, which prevents thrombotic events. However, upon stimulation with pro-inflammatory agents like tumor necrosis factor alpha (TNFα), CD40 ligand (CD40L), interleukin-1 beta (IL-1β), or histamine, ECs express tissue factor, resulting in activation of the coagulation cascade. Tissue factor, by inducing coagulation, seems to be involved in the pathogenesis of atherosclerosis and the initiation and propagation of acute coronary syndromes as well [45]. Elevated tissue factor level and activity have indeed been detected in patients with unstable angina. Moreover, tissue factor is highly expressed in atherosclerotic plaques in numerous cell types including ECs, smooth muscle cells, and monocytes/macrophages [46, 47]. However, additional studies are required to elucidate the role of tissue factor in the development of atherosclerosis *in vivo.*

Recently, it has been observed that histamine induces tissue factor expression in a concentration-dependent manner on both human ECs and VSMCs [48]. Histamine-induced tissue factor expression is mediated by activation of the H1 receptor, since

H1 receptor antagonists prevent this effect. Although mRNA stability, intracellular storage and localization of tissue factor may significantly modulate its activity, tissue factor induction in response to inflammatory stimuli is predominantly regulated at the transcriptional level. Functional studies indicate that Sp1 controls basal tissue factor gene expression, while a promoter (-227 to -172) containing two AP-1 sites and an NF-kappa B site mediates induction of tissue factor mRNA in monocytes and ECs [49]. Accordingly, tissue factor is activated by several signal transduction pathways including mitogen-activated protein kinases (MAPKs), and protein kinase C. Consistent with these data, p38, extracellular signal-regulated kinase and c-Jun N-terminal kinase inhibitors attenuate histamine-induced tissue factor expression, suggesting a crucial role of MAPK in this process (Figure 5.1.1) [48].

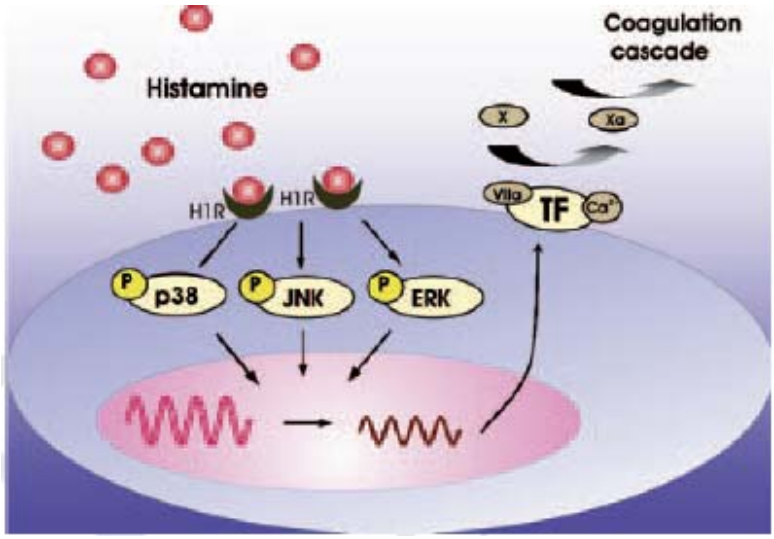

Figure 5.1.1 Histamine and thrombosis. Histamine induces tissue factor expression on both human ECs and VSMCs via activation of MAPKs. This histamine effect is mediated by histamine H1 receptor (H1R). Tissue factor expression leads to coagulation cascade activation and eventually thrombus formation. ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; TF, tissue factor.

5.2 Weibel-Palade Bodies

ECs contain rod-shaped storage vesicles called Weibel-Palade bodies (WPBs) harboring preformed vWF multimers and P-selectin. Stimulation of ECs with molecules raising intracellular Ca^{2+} levels like histamine or thrombin result in exocytosis of the WPB content to the EC surface (Figure 5.2.1). Histamine H1 receptor, but not H2 receptor antagonists abolish this effect [50].

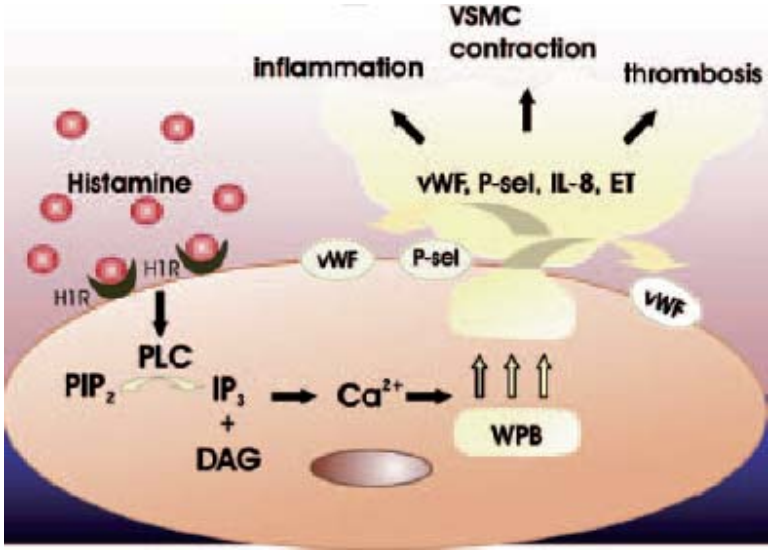

Figure 5.2.1 Histamine induces WPB release. Histamine induces exocytosis of endothelial WPBs in a Ca^{2+} - dependent manner. Released molecules like vWF, P - selectin (P-sel), IL–8 and endothelins (ET) modulate several important physiologic processes like inflammation, homeostasis and vasomotion. DAG, diacylglycerol; H1R, histamine H1 receptor; IP3, inositol-1,4,5-triphosphate; PIP2, phosphatidylinositol (4,5)-bisphosphate.

Both vWF and P-selectin are known to play a crucial role in thrombus formation. vWF is a large glycoprotein stabilizing coagulation factor VIII and participating in the binding of activated platelets to exposed collagen through receptors such as glycoprotein Ib on the platelet surface. Deficiency or mutation of vWF causes von

Willebrand disease, which is characterized by an increased bleeding time [51]. In contrast, P-selectin is a member of the selectin adhesion molecule family, which mediates initial rolling and attachment of leukocytes to the activated endothelium promoting the development of inflammation [52]. Recent studies, however, reveal additional possible role of P-selectin in thrombus formation. Binding of P-selectin to P-selectin glycoprotein ligand-1 indeed mediates the interaction of platelets with the endothelium as well as interaction of platelets with leukocytes and other platelets, thereby enhancing thrombotic events. What is more, P-selectin has been reported to enhance the expression of tissue factor on cultured human monocytes [53]. Consistent with these observations, P-selectin-deficient mice exhibit an abnormally prolonged bleeding time and unusual thrombus geometry during *ex vivo* thrombus formation [54]. In contrast, transgenic animals having high soluble P-selectin level exhibit a pro-coagulant state [55]. In humans, elevated P-selectin level is associated with prothrombotic disorders such as stroke or peripheral artery disease [56].

Hence, histamine induces a dose-dependent secretion of both vWF and P-selectin from WPBs, by a Ca^{2+} dependent mechanism, which can be blocked by mepyramine, a histamine H1 receptor antagonist. These observations strongly suggest a crucial role of histamine in thrombus formation by stimulating the release of WPBs.

5.3 Platelet aggregation

When vessel integrity is compromised, activated platelets bind to exposed collagen and vWF to form a hemostatic plug stabilized by fibrin. Similar to mast cells and other inflammatory cells, platelets contain intracellular granules containing numerous inflammatory and thrombogenic mediators like histamine, platelet factor 4, platelet-derived growth factor, ADP, and reactive oxygen species. Exposure of platelets to thrombin indeed results in aggregation and histamine release. The presence of histamine receptors on the platelet membrane, high activity of HDC, and the ability to take up preformed histamine suggest that histamine plays a role in platelet activation. Indeed, histamine enhances aggregation of isolated human platelets induced by a variety of agonists. The effect of histamine on platelet aggregation is abolished by H1 receptor, but not H2 receptor antagonists [57, 58]. On the other hand, histamine enhances eNOS activation resulting in endothelial NO production, which inhibits platelet activation and represents an important negative feedback mechanism [33, 35]. In conclusion, histamine plays a role not only in initiation of coagulation by stimulating tissue factor expression as well as WPBs release, but also regulates platelet aggregation during thrombus formation.

6. Inflammation

Histamine is a well-known mediator of inflammation and allergic responses. Released from mast cells or macrophages during inflammatory processes, it can affect immune responses at several levels. Most of the proinflammatory actions of histamine are mediated via the H1 receptor and activation of MAPKs as well as NFκB [59]. In contrast, the immunoregulatory effects of histamine are primarily mediated via the H2 receptor [60].

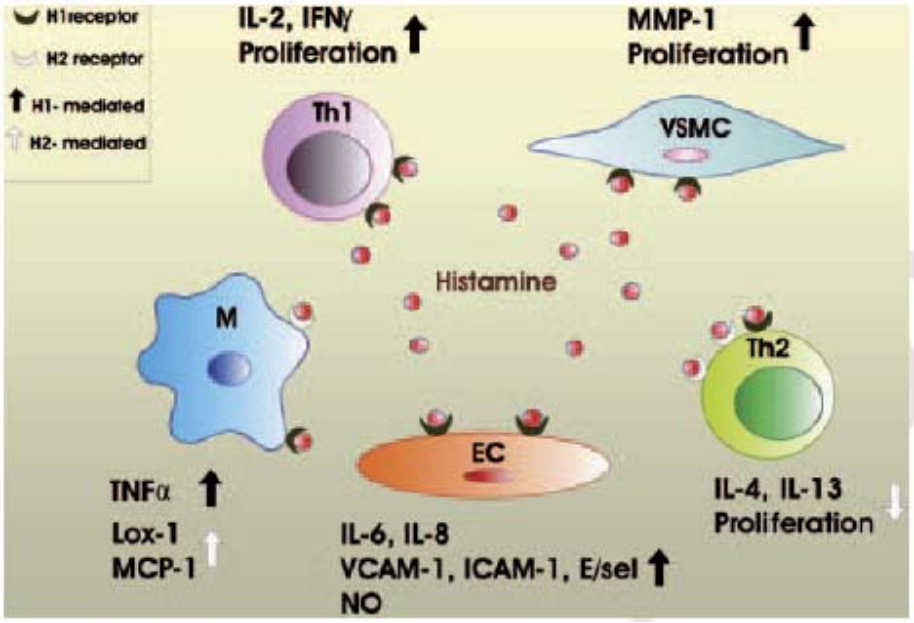

Figure 6.1 Histamine and inflammation. Histamine modulates expression of several proinflammatory molecules by different types of cells–monocyte/macrophages (M), Th1, Th2, ECs and VSMCs. E/sel. E-selectin; IFN, interferon; MMP, matrix metalloproteinase.

Histamine modulates progression of inflammation at many levels. It increases vascular permeability, enhances adhesion molecule expression, probably plays a

role in activation of monocytes/macrophages, modulates the Th1 versus Th2 response, and the production of pro- as well as anti-inflammatory cytokines. (Figure 6.1) [15].

6.1 Vascular Permeability

An increased permeability leading to tissue edema and accumulation of leukocytes is a hallmark of inflammation. Similarly, early atherosclerosis is characterized by the accumulation of low density lipoproteins in the arterial intima, occurring secondary to increased EC permeability. Histamine, acting via both H1 receptor and H2 receptor, is one of the most important regulators of this process. It has been observed that histamine causes transient phosphorylation of myosin light chains, actin cytoskeleton rearrangement, and intracellular gap formation in ECs occurring in a Ca^{2+}-dependent manner, which leads to increased vascular permeability [61, 62]. Inhibition of the MAPK kinase 1/2 with a specific peptide inhibitor significantly attenuates the histamine mediated changes in venular permeability [63].

6.2 Adhesion Molecule Expression

Under normal conditions, the endothelial layer resists prolonged contact with blood leukocytes. Upon activation, however, ECs start to express molecules, which facilitate firm adhesion and extravasation of leukocytes into sites of inflammation. This process is regulated by different families of adhesion molecules expressed on both, leukocytes and ECs. The types of adhesion molecules and their interaction regulate the specificity of leukocyte migration into tissues. The selectin family of

adhesion molecules (E-, P-, and L-selectin) mediates the initial attachment of leukocytes to the endothelial layer before firm adhesion mediated via integrins, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule (ICAM), and platelet EC adhesion molecule-1 (CD31) occurs. Genetic deletion of either E-selectin, P-selectin, or VCAM-1 significantly diminishes the formation of atherosclerotic lesions. Histamine, acting via H1 receptor and NFκB, enhances IL-4 and TNFα-mediated expression of VCAM-1, TNFα- mediated expression of E-selectin and ICAM-1, as well as surface translocation of P-selectin in human ECs *in vitro* [64, 65]. These observations suggest that histamine plays an important role in regulating adhesion of leukocytes to the activated endothelium. Indeed, in a rat model, histamine induces rolling of polymorphonuclear cells in a P-selectin dependent manner, which is almost completely abolished by H1 receptor, but not H2 receptor blockade [66, 67].

6.3 Leukocyte Accumulation

Leukocyte accumulation is controlled by chemoattractant molecules and adhesion molecules, and represents an important process in atherosclerosis, leading to plaque progression. Adherent leukocytes enter the intima by diapedesis. Histamine enhances expression of several chemoattractant cytokines such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) [68, 69]. *In vivo* studies demonstrate the importance of these molecules: mice lacking MCP-1 or its receptor-chemokine (C-C motif) receptor 2 exhibit a striking decrease in mononuclear phagocyte accumulation and atherosclerotic plaque development.

6.4 Regulation of Th1/Th2 Balance

Many inflammatory processes are associated with accumulation of $CD4^+$ T cells subsets. Stimulated by antigen, $CD4^+$ T cells can differentiate into two types of effector cells, namely Th1 and Th2. In general, Th1 cells activate monocytes/macrophages and induce B cells to produce IgG antibodies; in contrast Th2 cells initiate the humoral immune response by activating naive antigen-specific B cells to produce IgM antibodies. The immune responses enhancing Th1 effector mechanisms are considered to be pro-atherogenic. Th1 and Th2 cells exhibit differential expression of histamine receptors - Th1 cells preferentially express H1 receptor, whereas Th2 cells show predominant expression of H2 receptor. Stimulation of Th1 cells, but not Th2 cells, with histamine results in increased intracellular calcium level, translocation of NF-AT, and activation of IL-2. Indeed, histamine enhances CD3-induced proliferation of Th1 cells and blocks proliferation of Th2 cells. This effect of histamine is abolished by H1 receptor or H2 receptor blockers, respectively. T cells lacking the H1 receptor exhibit significant suppression of interferon-γ secretion, enhancement of Th2 cytokine production - IL-4 and IL-13 - and reduction of T-cell proliferation. In contrast, T cells lacking H2 receptor exhibit significant enhancement of both Th1- and Th2-specific cytokines [60]. These observations suggest that histamine can modulate T cell-mediated immune responses and thereby the development of atherosclerosis and other diseases.

6.5 Macrophage Activation

Macrophage-derived foam cells are one of the most abundant components of atherosclerotic plaques. Foam cells do not only scavenge modified low-density lipoprotein particles rich in cholesterol, but also express many proinflammatory molecules. Monocytes switch their receptor expression profile from H2 receptor to H1 receptor upon stimulation, suggesting that histamine signaling may influence the transition from monocytes to activated macrophages [70]. Moreover, histamine, acting via the H2 receptor, enhances expression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), which scavenges modified lipoprotein particles, and that of the chemoattractant MCP-1, thereby possibly enhancing both macrophage accumulation and foam cell formation [71]. Accordingly, histamine mediated upregulation of both LOX-1 and MCP-1 is abrogated in activated macrophages which express predominantly H1 receptor.

6.6 Obesity

Obesity is an important risk factor for the development of cardiovascular diseases. Obesity is regulated by both, environmental and genetic factors. Leptin, the product of the ob gene, exerts wide-ranging effects including food intake and energy expenditure. Consistently, disruption of leptin protein or leptin receptor leads to the development of obesity. Interestingly, hypothalamic neuronal histamine contributes to the leptin signaling pathway via the H1 receptor. Indeed, mice with targeted deletion of H1 receptor exhibit a disrupted diurnal feeding rhythm, increased food consumption, and with time develop obesity [18]. In contrast, H2 receptor blockers

improve excess weight in patients with type 2 diabetes mellitus as well as in patients treated with anti-psychotic drugs [72].

7. Atherosclerosis

Complications of atherosclerosis are the leading cause of death in Western societies. Atherosclerosis is a progressive inflammatory arterial disease characterized by accumulation of lipids and leukocytes, proliferation of VSMCs, and excessive production of extracellular matrix. The first stage of atherosclerosis development is characterized by activation of the endothelial layer lining the blood vessels, leading to functional activation, increased permeability and recruitment of leukocytes. The first cells found in the intima are macrophages, which scavenge oxidized low density lipoproteins leading to lipid loaded foam cell formation. Foam cells express numerous cytokines, metalloproteinases, and reactive oxygen species, which all promote development of inflammation and progression of atherosclerosis. Advanced plaques with a necrotic core composed of monocytes, foam cells, and lipids as well as a fibrous cap consisting of connective tissue is a hallmark of advanced atherosclerosis leading to narrowing of the vessel and clinical manifestations [73].

Many cell types involved in development and progression of atherosclerosis, such as ECs, macrophages, mast cells, and VSMCs are able to produce histamine and express histamine receptors. Indeed, HDC, the rate limiting enzyme for histamine production, is highly upregulated in murine atherosclerotic lesions [3]. Moreover, numerous processes crucial for atherosclerosis development such as vascular permeability, adhesion molecules expression, leukocyte activation, and lipid metabolism are known to be affected by histamine. These observations strongly

suggest that histamine modulates the development and progression of atherosclerosis, and pharmacological or genetic blockade of histamine receptors may have beneficial effects in this context.

8. Autoimmune Diseases and Allergy

8.1 Autoimmune Diseases

The incidence of autoimmune diseases has increased significantly during the last years in developed countries. The reason for this phenomenon, however, is not clear. Under normal conditions, the immune system is able to distinguish between self components and external pathological antigens since self-tolerance is controlled by clonal selection of T and B cells during their maturation. Autoimmune disease occurs when this normal state of self-tolerance is altered, for example, when antigen is not abundant/organ specific, or not expressed in thymus, or when tolerance to an abundant antigen is broken. Autoimmune diseases result from a disrupted balance between anti- and pro-inflammatory signaling. Hence, degranulation of mast cells and the resulting increase in plasma histamine levels has been reported during the development of autoimmune diseases [74, 75]. Indeed, due to its modulating effect on immune responses, histamine receptors are interesting targets in therapy of autoimmune diseases.

8.2 Allergy

Histamine is an important mediator of allergic disease, as it is mainly stored in mast cells and basophils. Allergic reactions can be induced when allergens cross-link IgE bound to the FcεRI receptor on mast cells, but several other stimuli such as cytokines and complement components can induce activation of mast cells as well. Activated mast cells are involved in inflammation by secreting molecules stored in

their granules such as histamine as well as by producing cytokines and leukotrienes. The release of inflammatory mediators, cytokines, and chemokines at sites of allergic reactions results in the recruitment of eosinophils and basophils. Overall, leukocyte activation provokes a variety of inflammatory symptoms from sneezing and a running nose to life threatening bronchoconstriction, hypotension and shock depending on the dose of allergen and the route of entry. The inflammatory reaction triggered by mast cell activation can be classified as an immediate response, occurring within minutes after mast cell activation, and a late-phase response lasting hours or days. Degranulation of mast cells leading to the release of histamine, prostaglandins, and other molecules enhancing vascular permeability, and inducing inflammation is a main mediator of the immediate response. The late phase response is generated via production of proinflammatory cytokines, chemokines, and leukotrienes.

During experimental asthma, allergic rhinitis, and in patients with urticaria, histamine level is significantly higher when compared with healthy subjects. Indeed, it has been demonstrated that histamine is affecting both immediate and late phase allergic responses [1, 76].

8.2.1 Immediate Response

Mast cells and basophils are the main sources of stored histamine. Activation of these cells results in degranulation and a sudden transient increase in histamine concentration. Hence, release of stored histamine from these cells accounts for histamine mediated immediate allergic reactions. Acting via the H1 receptor,

histamine increases vascular permeability leading to edema, bronchoconstriction, and mucus production. These alterations result in the development of typical allergic symptoms such as bronchial obstruction, sneezing, rhinitis, and itching. Consistent with the pathological role of the H1 receptor, administration of histamine H1 receptor blockers is known to have beneficial effects on such responses.

8.2.2 Long-Term Response

Histamine is also involved in the regulation and modulation of long-term inflammatory responses. Recent studies demonstrate that macrophages and neutrophils are the main source of inducible histamine. Many cell types involved in the development of allergic responses are known to express histamine receptors on their surface. Neutrophils and eosinophils express both H1 receptor and H2 receptor, whereas basophils express predominantly H2 receptor. In contrast to the immediate response, where histamine promotes the allergic reaction, late allergic response mediated by basophils and neutrophils is rather attenuated by histamine. Indeed, histamine, acting via H2 receptor, suppresses leukotriene synthesis [77]. This observation confirms the ambiguous role of histamine in the development of inflammation and allergy.

9. Conclusions

Histamine is an endogenous amine produced in a one step reaction from L-histidine. Activity of HDC, a rate limiting enzyme crucial for histamine formation, is detected in many different cell types including various cells of the immune system, gastrointestinal and bronchial endocrine cells, neuroendocrine cells, as well as some tumor cells.

Histamine mediates several processes like homeostasis, vasomotion, and inflammation crucial for normal organism function. The action of histamine depends on several factors such as its concentration, the type of receptor it activates, and the vascular bed it acts on.

Elevated plasma and/or tissue histamine levels are often associated with pathogenesis of cardiovascular, allergic, and autoimmune diseases. Indeed, histamine is known to modulate numerous processes such as vascular permeability, adhesion molecule expression, and leukocyte activation, which are crucial for the development of atherosclerosis, allergy, or autoimmune disease. Therefore, pharmacological or genetic blockade of histamine receptors, for some of these disorders, represent a well established, for others a promising therapy.

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3.4 Role of L-selectin in atherogenesis

Atherosclerosis is a chronic inflammatory disease resulting from accumulation of cholesterol and leukocytes into the arterial intima. Several molecules regulate extravasation of inflammatory cells. Multiple studies indicate that the initial phase of this process is mediated primarily by the selectin family of adhesion molecules [18, 28-30]. Indeed, it was demonstrated *in vivo* that the capture and rolling of leukocytes along activated endothelium are severely compromised in animals lacking either of the selectins [28, 30-32].

L-sel is expressed ubiquitously by most leukocytes, where it serves adhesive as well as signaling functions [33, 34]. Like other members of the selectin family, L-sel contains an extracellular region that includes a C-type lectin domain, an epidermal growth factor like domain, and short consensus repeat units homologous to domains found in complement-binding proteins [35].

Molecules such as c-jun n-terminal kinase (JNK) and NFκB influence a variety of pro-atherogenic processes, such as EC activation, T-cell differentiation, proliferation, and migration of VSMCs. It has been described that mice lacking JNK-2 show decreased atherosclerosis due to reduced oxLDL uptake by macrophages [36]. L-sel does not only play an important role in leukocyte homing and extravasation, but it is also involved in the regulation of signal transduction via its cytoplasmic domain. Indeed, ligation of L-sel with a specific antibody or ligand elicits a number of intracellular events including activation of JNK, NFκB, as well as protein kinase C, and leads to leukocyte activation [33, 34].

The ligands of L-sel vary depending on the vascular bed and the type of blood vessel. Recently, it has been proposed that activated endothelium lacks functional L-sel ligand [37]. Instead, L-sel exerts its physiological functions due to its interaction with P-sel glycoprotein ligand-1 expressed by the leukocytes. This finding emphasizes the importance of secondary capture resulting from leukocyte-leukocyte interaction [38].

L-sel mediates interactions of lymphocytes with high endothelial venules of peripheral lymph nodes which are crucial for lymphocyte homing and activation, and it is required for the initial stages of leukocyte extravasation into sites of inflammation. Indeed, mice lacking *L-sel* exhibit drastically reduced lymphocyte homing to peripheral lymph nodes as well as significantly reduced leukocyte capture and rolling across activated endothelium [18, 28, 38]. Moreover, it has been demonstrated that lymphocyte recruitment into the aortic wall during atherosclerosis development is partially L-sel dependent [39].

Although the role of E- and P-sel in atherogenesis is well known [40], there are no in vivo studies addressing the role of L-sel in the development and progression of atherosclerosis in vivo.

3.5 Role of lectin-like oxLDL receptor-1 in atherogenesis

LOX-1, together with SR-A and CD36, belongs to the family of scavenger receptors [41]. Chemically, it is a type II glycoprotein with short cytoplasmic N-domain, a transmembrane domain, and an extracellular lectin-like domain [13]. Homodimerisation of LOX-1 assures optimal ligand binding [42]. LOX-1 binds

several ligands including advanced glycation-end products, modified lipoproteins, apoptotic bodies as well as bacterial components. Expression of LOX-1 upon normal conditions is usually low and increases during pathological conditions like elevated blood pressure, diabetes, or atherosclerosis [43-45]. Several mediators such as oxLDL, angiotensin II, cytokines, sheer stress, and advanced glycation end-products have been shown to modulate LOX-1 expression [46-50]. Unlike SR-A and CD36, LOX-1 is the main scavenger receptor expressed by ECs [13, 45]. In addition, several studies prove the presence of LOX-1 on monocytes/macrophages, VSMCs, and platelets (Fig. 4); under these conditions, however, its role seems to be secondary to that of other scavenger receptors [41].

Ligation of LOX-1 results not only in oxLDL uptake, but also in activation of molecular pathways leading to increased production of ROS, NFκB activation, and, as a result, ECs dysfunction [50, 51]. LOX-1-mediated signaling leads to increased production of adhesion molecules (E-sel, P-sel, and VCAM-1) and chemokines (MCP-1), which has been shown to be important for leukocyte migration into sites of inflammation [52-54]. LOX-1 ligation also leads to diminished NO production and increased metalloproteinase expression that promote EC dysfunction [55, 56]. Moreover, increased ROS release generates a positive feedback loop for further LDL oxidation. Indeed, it has been shown that mice lacking *LOX-1* exhibit significant reduction in atherosclerotic plaque formation due to reduction in proinflammatory and prooxidant signals [57].

Recent reports demonstrate the important role of LOX-1 in both early and advanced atherosclerosis. However, the relevance of endothelial LOX-1 in the pathogenesis of atherosclerosis remains unclear.

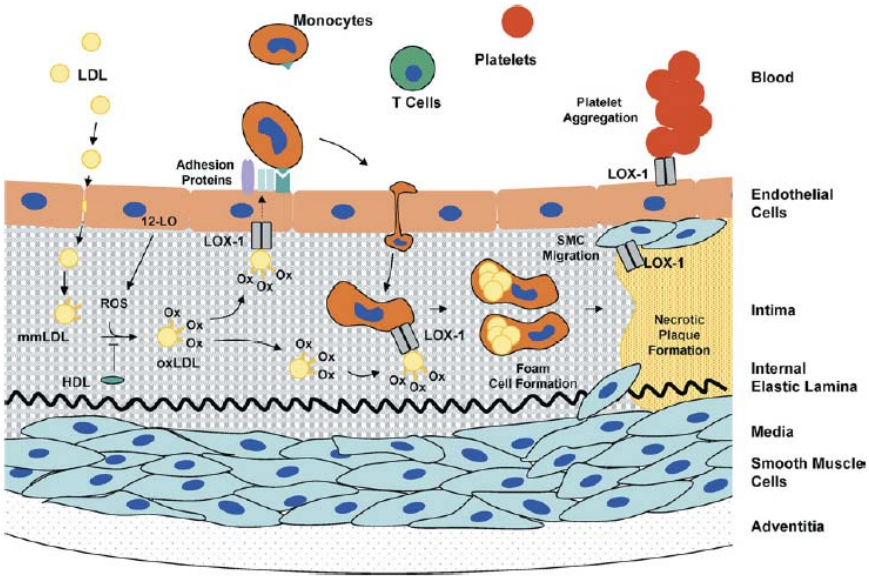

Figure 4

Expression of LOX-1 by cellular components of atherosclerotic plaque; adapted form Dunn et al [44].

4 The goal of the study

The aim of the study was to find and characterize new molecules relevant for atherosclerosis development.

5 Animal models used in the study

5.1 *Apolipoprotein E* deficient mice

Mice are the most commonly used model to study the causal relation between genes and the phenotype of human diseases. However, wild-type mice are resistant towards atherosclerosis development due to the very low levels of plasma LDL and relatively high levels of high density lipoproteins (HDL). Therefore, genetically modified animals have to be ussed. Developed in the laboratory of Dr. Nobuyo Maeda at The University of North Carolina, *ApoE*-deficient mice are a widely used model of atherogenesis [58, 59]. ApoE is part of the LDL particle recognized by the LDL receptor (LDLr). Its deficiency significantly increases plasma LDL levels, whereas plasma HDL remains unchanged (Tab. 1) [60]. Thus, animals lacking a functional *ApoE* gene develop atherosclerosis spontanously starting from approximately the 3rd month of age [59].

	WT normal diet	***ApoE*$^{-/-}$ normal diet**	***ApoE*$^{-/-}$ atherosclerotic diet**
Number of mice	12	12	11
Total cholesterol (mg/dl)	51.8 ± 6.2	142.2 ± 37.3*	359.9 ± 91.4*
HDL cholesterol (mg/dl)	33.3 ± 6.0	29.9 ± 6.4	33.4 ± 12.4
LDL cholesterol (mg/dl)	13.6 ± 5.4	101.2 ± 29.3*	318.2 ± 72.8*

Table 1

Plasma lipids in WT and *ApoE*$^{-/-}$ mice. Data expressed as mean ± SD. *ApoE*$^{-/-}$ normal vs. WT mice (*P<0.05), *ApoE*$^{-/-}$ atherosclerotic vs. normal diet mice and WT mice (*P<0.05), *ApoE*$^{-/-}$ normal diet vs. WT mice (*P<0.05); adapted from Guo et al [60].

5.2 Histamine *H1 receptor* and *H2 receptor* deficient mice

H1R$^{-/-}$ mice have been developed by homologous recombination in 1996 at the Department of Molecular Immunology at Kyushu University in Japan in order to study the effect of histamine signaling on behavior and activity. Indeed, animals lacking *H1R* exhibit significant impairment of locomotor and exploratory behavior. *H1R* deficient mice develop obesity starting from 48 weeks of age [61]. Moreover, these animals exhibit altered immunological response to various stimuli which make them very interesting targets to study in the context of atherosclerosis development [62, 63]. *H2R*$^{-/-}$ mice have been created in year 2000 in the same laboratory to investigate the effect of histamine signaling on gastrointestinal functions. The study of Kobayashi et al. demonstrated that,

although histamine acting via H2R regulates gastric acid secretion, deletion of *H2R* does not impair secretion of gastric acid [64]. A later study showed that deletion of *H2R* results in attenuation of Th1 effector cell responses and susceptibility to experimental allergic encephalomyelitis [65].

5.3 *L-selectin* deficient mice

Mice lacking *L-sel* have been generated by homologous recombination at the Department of Immunology of the Duke University, Medical Center Durham, North Carolina, USA, , to study the relevance of L-sel in leukocyte homing to peripheral lymph nodes, interactions between leukocytes and activated vascular endothelium, as well as leukocyte distribution.

L-sel$^{-/-}$ mice are viable and fertile, however, they exhibit abnormal leukocyte distribution, as well as leukocyte rolling along activated endothelium [28].

5.4 *Lectin-like oxLDL receptor-1* transgenic mice

Transgenic mice overexpressing LOX-1 specifically in ECs were developed in our laboratory by Dr *Alexander* Akhmedov using the EC-specific Tie2 promoter. Here, we report for the first time the phenotype of these animals.

6. Summary of the results

6.1 "Histamine H1 receptor promotes atherosclerotic lesion formation by increasing vascular permeability for low density lipoproteins"

A) Pharmacological blockade or genetic deletion of *H1R*, but not *H2R*, decreases atherosclerosis development in *ApoE*$^{-/-}$ mice.

B) Atherosclerotic animals lacking *H1R* exhibit decreased arterial permeability to LDL.

C) Accumulation of lipids and leukocytes into atherosclerotic plaques is diminished in the absence of *H1R*.

D) Spleen size and the number of blood lymphocytes are decreased in the absence of *H1R*.

E) Production of Th1-specific cytokines: IL-2, IFNγ, and CCL5 (but not Th2-specific cytokines: IL-4 and IL-10) is diminished in the spleen of *ApoE*$^{-/-}$ *H1R*$^{-/-}$ mice.

F) H1R expressed on vascular cells determines the development of atherosclerosis.

G) H1R expressed on bone-marrow derived cells does not affect atherosclerosis.

6.2 "Accelerated early atherosclerosis in mice deficient in *L-selectin*"

A) Genetic deletion of *L-sel* increases early but not advanced atherosclerosis in *ApoE*$^{-/-}$ mice.

B) *L-sel* deletion does not influence leukocyte capture and rolling along aorta during atherosclerosis development.

C) *L-sel* deletion does not influence cellular composition of atherosclerotic plaque in *ApoE*$^{-/-}$ mice.

D) *L-sel* deletion results in altered blood lymphocyte numbers in *ApoE*$^{-/-}$ mice.

E) The presence of L-sel is necessary for maintaining proper leukocyte distribution within the body.

6.3 "Endothelial overexpression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) increases LDL uptake and plaque formation"

A) Endothelial-specific overexpression of LOX-1 results in accelerated atherosclerosis in both *LOX-1 TG* and *LOX-1 TG*/*ApoE*$^{-/-}$ animals.

B) ECs overexpressing LOX-1 exhibit a significant increase in oxLDL uptake.

C) Endothelial-dependent relaxation to acetylcholine is impaired upon LOX-1 overexpression.

D) Both *LOX-1* TG and *LOX-1 TG*/*ApoE*$^{-/-}$ mice exhibit significantly increased expression of adhesion molecules.

E) *LOX-1 TG* animals exhibit a significant increase in accumulation of leukocytes into atherosclerotic plaques.

7. Publications that contributed to the work

“Histamine H1 receptor promotes atherosclerotic lesion formation by increasing vascular permeability for low density lipoproteins”. Manuscript submitted.

Own contribution:

1) Animal breeding, generation of *ApoE*$^{-/-}$ *H1R*$^{-/-}$ and *ApoE*$^{-/-}$ *H2R*$^{-/-}$ mice
2) Animal experiments
    - Harvesting of the mice
    - En-face Oil-red O staining of aortas
    - Quantification of atherosclerosis
3) Immunohistochemistry
    - Cryosection of aortic root, spleen and lymph node
    - Immunostainings
    - Analysis of immunostainings (AnalySIS-Five)
4) FACS analysis of murine splenocytes (together with Pavani Mocharla)
5) Isolation of mRNA and protein from mouse aorta and spleen
6) qRT PCR
7) Bone marrow transplantation (together with Janin Hofmann)
8) Proliferation of VSMC *in vivo*, 5’-bromo-2’-deoxyuridine (BrdU) incorporation
9) *In vivo* permeability assay using EBD and I^{125} LDL.

Histamine H1 Receptor Promotes Atherosclerotic Lesion Formation by Increasing Vascular Permeability for Low Density Lipoproteins

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Nonstandard abbreviations

ApoE:	apolipoprotein E
CCL5:	chemokine (C-C motif) ligand 5
EBD:	evans blue dye
HCD:	high cholesterol diet
H1R:	histamine H1 receptor
H2R:	histamine H2 receptor
LDL:	low-density lipoprotein
ORO:	oil red-o
PFA:	paraformaldehyde
SMa:	α-smooth muscle actin
qRT PCT:	quantitative real time polymerase chain reaction
VSMC:	vascular smooth muscle cell

Abstract

Enhanced endothelial permeability with intimal accumulation of low density lipoproteins (LDL) stimulates the formation of atherosclerotic lesions. Histamine is known to increase vascular permeability. Here we demonstrate that *ApoE*$^{-/-}$ mice treated with a histamine H1, but not H2 receptor antagonist developed 40% less atherosclerotic lesions in the aorta than placebo treated controls. Similarly, genetic deletion of the H1, but not H2 receptor resulted in 60% reduction of lesions compared to *ApoE*$^{-/-}$ controls. The H1 receptor enhanced permeability for LDL and lipid accumulation in aorta, whereas plasma lipoprotein levels remained unaffected. In contrast, the H1 receptor did not affect proliferation and migration of vascular smooth muscle cells. Bone marrow transplantation confirmed that the formation of atherosclerotic lesions depended on the H1 receptor on vascular cells, while its presence on bone marrow-derived cells was irrelevant for plaque development. Mice expressing the H1 receptor exhibited higher levels of the chemokine CCL5 and higher numbers of macrophages and lymphocytes in plaques, higher numbers of circulating lymphocytes, and larger spleens. These observations indicate that H1, but not H2 receptor activation drives the formation of atherosclerotic lesions through an increased vascular permeability for LDL, which is associated with an enhanced secondary aortic and systemic inflammation.

Introduction

Histamine (2-[4-imodazole]-ethylamine) is a biogenic amine involved in inflammatory responses and plays a dominant role in acute allergic reactions (1, 2). In this context, it increases vascular permeability resulting in the formation of erythema and edema. In the arterial system, histamine has been shown to participate in the response to acute injury by promoting arterial remodeling and enhancing neointima formation (3). There is increasing evidence that histamine may also modulate the response to chronic arterial injury such as atherosclerotic lesion formation (3-5). An enhanced vascular permeability is indeed an early alteration of arterial function during the formation of such lesions. This increase in permeability is an important prerequisite for the accumulation of low density lipoproteins (LDL) in the evolving lesions (6-8). Once LDL are present in the vessel wall, they become oxidized and thereby exhibit chemotactic and pro-inflammatory properties leading to monocyte accumulation, foam cell formation, and progression of lesion formation (9). However, whether histamine enhances vascular permeability in the setting of chronic arterial injury and whether such an effect would promote the formation of atherosclerotic lesions has not been examined yet.

Histamine exhibits its actions through four G-protein coupled membrane receptors, of which the H1 and H2 receptor are expressed in the vessel wall (4). Most of the deleterious vascular effects of histamine such as vasospasm, tissue factor induction, and intimal thickening after acute arterial injury are mediated by the H1 receptor (3, 10-13); similarly, the increase in vascular permeability induced by histamine is mediated via this receptor (14, 15). These observations indicate an important role of the H1 receptor in the vascular response to acute injury. This study was therefore designed to investigate using a combined pharmacological and genetic approach whether (1) histamine promotes atherosclerotic lesion formation, (2) whether an increased vascular permeability is involved, and (3) whether these effects are mediated by the H1 receptor.

Results and Discussion

Histamine H1 receptor promotes the formation of atherosclerotic lesions

To assess the role of histamine receptor activation in the formation of atherosclerotic lesions, 6 week old mice were fed a high cholesterol diet for 12 weeks and treated with the H1 receptor antagonist mepyramine or the H2 receptor antagonist ranitidine throughout this time period. Animals treated with mepyramine, but not ranitidine, exhibited a nearly 40% reduction in atherosclerotic lesions (mepyramine: $p<0.05$ vs. control; ranitidine: p=NS vs. control; Fig 1A and B). This observation was confirmed by a genetic approach involving *ApoE / H1 receptor* double knockout (*ApoE*$^{-/-}$*H1R*$^{-/-}$) and *ApoE / H2 receptor* double knockout (*ApoE*$^{-/-}$*H2R*$^{-/-}$) animals. *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice exhibited a 60% decrease in atherosclerotic lesions as compared to *ApoE*$^{-/-}$ controls ($p<0.01$; Fig 1C and D). In contrast, plaque formation in *ApoE*$^{-/-}$*H2R*$^{-/-}$ mice did not differ from *ApoE*$^{-/-}$ controls (p=NS; Fig 1C and D). It is not surprising that genetic deletion of the H1 receptor had a more pronounced effect on plaque formation than pharmacological blockade, as this difference is consistent with the higher efficacy of the genetic as compared to the pharmacological approach. These observations demonstrate that activation of the H1 receptor, but not the H2 receptor, promotes the formation of atherosclerotic lesions. Hence, H1 receptor activation is involved in the vascular response to chronic injury.

Plasma levels of cholesterol and triglycerides as well as the respective subfractions did not differ in *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice and *ApoE*$^{-/-}$ controls (p=NS; Tab 1), indicating that the effect of H1 receptor activation does not depend on plasma lipoproteins. The H1 receptor thus modulates vascular lesion formation independent of changes in lipid metabolism. Although food intake was similar in the different groups of animals (Tab 1), *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice were slightly heavier than *ApoE*$^{-/-}$ controls, which may be related to the known reduced physical activity of *ApoE*$^{-/-}$*H1R*$^{-/-}$ animals (16). However, the weight difference between the two groups of mice likely did not affect the formation of atherosclerotic lesions, since the heavier group would be expected to exhibit

more rather than less lesions (17).

The expression of histidine decarboxylase, H1 receptor, and H2 receptor in mouse aorta was assessed using qRT-PCR. Histidine decarboxylase expression was 2.5-fold higher in aorta of atherosclerotic *ApoE*$^{-/-}$ animals as compared to healthy age-matched wild-type controls (n=6-8; $p<0.05$; data not shown). In contrast, the formation of atherosclerotic lesions did not affect aortic expression of H1 and H2 receptor (n=6-8; p=NS; data not shown). Thus, the development of atherosclerosis by itself did not interfere with the target of the experimental intervention.

Histamine H1 receptor increases endothelial permeability to LDL

Histamine is known to increase vascular permeability in acute allergic reactions via the H1 receptor (14, 18-21). Enhanced endothelial permeability for LDL is a very important feature of atherosclerotic lesion formation (6, 9); however, it is not known whether histamine receptor activation is involved. Here we observed that ^{125}I-LDL levels were more than 2-fold higher in the aortic wall of 18 week old *ApoE*$^{-/-}$ animals fed a high cholesterol diet as compared to age- and diet-matched *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice ($p<0.05$; Fig. 2A left panel), while simultaneously measured ^{125}I-LDL plasma levels did not differ (p=NS; data not shown). These mice exhibited pronounced atherosclerotic changes in their descending aortas as assessed by ORO en face staining (Fig. 2A right panel). The ORO-positive area in aortic cross sections at the level of the aortic valve was 24.9±3.2% and 16.5±1.7% of plaque area in *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice, respectively ($p<0.05$; Fig. 2B). Similar to mice on a high cholesterol diet, 18 week old *ApoE*$^{-/-}$ animals fed a normal diet showed more than 1.5-fold higher ^{125}I-LDL levels in the aortic wall as compared to *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice ($p<0.05$; Fig. 2C left panel), while while simultaneously measured ^{125}I-LDL plasma levels did not differ (p=NS; data not shown). These mice did not exhibit any atherosclerotic changes in their descending aortas as assessed by ORO en face staining (Fig. 2C right panel).

Hence, the H1 receptor enhances aortic permeability for LDL, which occurs both in the

presence and absence of atherosclerotic plaques. These observations indicate that activation of the H1 receptor causes a primary alteration of vascular function consisting in an enhanced permeability for LDL and in turn on increased intimal accumulation of these lipoproteins and increased formation of atherosclerotic lesions.

Histamine H1 receptor does not affect VSMC proliferation

VSMC proliferation contributes to the progression of atherosclerotic lesions. BrdU incorporation over a time period of 7 days was applied to monitor VSMC proliferation during atherosclerosis progression in mice fed a high cholesterol diet for 8 weeks. BrdU-positive cells occupied 1.4±0.2% of plaque area in *ApoE*$^{-/-}$ and 1.6±0.2% in *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice (p=NS; data not shown). Aortic tissue sections were double-stained with anti-smooth muscle alpha-actin to specifically investigate the proliferation of VSMC. The number of double positive cells normalized to plaque area was $6.5x10^{-5}$±$1.1x10^{-5}$ cells/μm^2 in *ApoE*$^{-/-}$ and $5.0x10^{-5}$±$0.7x10^{-5}$ cells/μm^2 in *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice (p=NS; Fig. 3A). In line with this, the total area occupied by smooth muscle alpha-actin positive cells did not differ in the two groups of animals (p=NS; Fig. 3B and C).

These observations indicate that the H1 receptor does not affect VSMC proliferation during the formation of atherosclerotic lesions. Both VSMC proliferation and VSMC positive plaque area were similar, suggesting that the H1 receptor does not affect VSMC migration either. Therefore, the H1 receptor does not promote the formation of atherosclerotic lesions via proliferation or migration of VSMC. Histamine was observed to stimulate proliferation of cultured VSMC (22); however, these experiments were performed *in vitro* and do not reflect the complex events occurring during plaque formation in vivo. Although VSMC do not contribute to the effect of the H1 receptor on plaque formation via proliferation or migration, they may still be involved in lesion progression via their metabolic functions.

Histamine H1 receptor on vascular, but not on bone marrow derived cells promotes the formation of atherosclerotic lesions

Vascular inflammation is an important event in the formation of atherosclerotic plaques. To determine whether the effect of the H1 receptor on lesion formation is mediated by vascular or by cells of the hematopoetic system, bone marrow transplantation experiments were performed. No difference in plaque formation was observed when reconstitution of *ApoE*$^{-/-}$ recipient mice with *ApoE*$^{-/-}$ bone marrow (8.0±1.0% aortic plaque area) was compared to that of *ApoE*$^{-/-}$ recipients with *ApoE*$^{-/-}$*H1R*$^{-/-}$ bone marrow (6.4±0.6%; p=NS; Fig. 4). Similarly, plaque formation did not differ when *ApoE*$^{-/-}$*H1R*$^{-/-}$ recipient mice were reconstituted with *ApoE*$^{-/-}$ bone marrow (3.3±1.1% aortic plaque area) or with *ApoE*$^{-/-}$*H1R*$^{-/-}$ bone marrow (2.6±0.3%; p=NS; Fig. 4). In contrast, *ApoE*$^{-/-}$ recipients exhibited significantly more atherosclerosis as compared to *ApoE*$^{-/-}$*H1R*$^{-/-}$ recipients irrespective of whether the mice were reconstituted with *ApoE*$^{-/-}$ or *ApoE*$^{-/-}$*H1R*$^{-/-}$ bone marrow ($p<0.01$ for *ApoE*$^{-/-}$ recipients vs *ApoE*$^{-/-}$*H1R*$^{-/-}$ recipients with either type of bone marrow for reconstitution; Fig 4).

These data indicate that the H1 receptor expressed on vascular cells rather than on bone marrow derived cells promotes the formation of atherosclerotic lesions. The data are in line with the observation that the H1 receptor enhances vascular permeability for LDL, since the latter is primarily regulated by vascular cells. This notion supports the concept that the increased vascular permeability is the driving force for plaque formation through the H1 receptor. Thus, histamine promotes plaque formation via the H1 receptor on vascular cells only, although bone marrow derived cells still participate in the formation of atherosclerotic lesions and may even release histamine under these conditions. Indeed, a reduced neointimal thickening after acute vascular injury was demonstrated in mice lacking L-histidine decarboxylase, the rate-limiting enzyme for histamine synthesis, and bone marrow derived cells expressing the enzyme enhanced neointima formation as compared to cells lacking it. These data indicate that bone marrow derived cells are a source of histamine in vivo suggesting that they may release

histamine during the formation of atherosclerotic lesions as well. In this case, however, the cells would contribute to plaque formation by releasing histamine, but not by responding to it.

Increased aortic and systemic inflammation in the presence of the H1 receptor

In *ApoE*$^{-/-}$ mice fed a high cholesterol diet for 12 weeks, immunohistochemistry revealed more CD68^{+} cells in plaques compared to *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice, indicating that the lesions contained a higher number of macrophages in the presence of the H1 receptor ($p<0.01$; Fig. 5A). A similar pattern was observed with CD3^{+} T lymphocytes ($p<0.01$; Fig. 5A) as well as with CD4^{+} T helper cells ($p<0.05$; Fig. 5A), while the number of CD8^{+} cytotoxic T cells did not differ (p=NS; Fig. 5A). Expression of the chemokine CCL5 (RANTES) was higher in plaques of *ApoE*$^{-/-}$ compared to *ApoE*$^{-/-}$ *H1R*$^{-/-}$ mice both at the mRNA and the protein level ($p<0.01$; Fig. 5B).

The higher number of macrophages and T lymphocytes in atherosclerotic lesions of mice expressing the H1 receptor indicates that inflammation of plaques is more active in the presence of the receptor. The enhanced levels of the potent chemokine CCL5 may well account for the higher number of leukocytes in these plaques. These data are in line with the concept that the increased vascular permeability for LDL is the driving force for plaque formation through the H1 receptor. When LDL accumulate in the vessel wall, they become oxidized and thereby highly chemotactic for leukocytes (9, 23-25); in addition, they induce the production of CCL5 (26, 27), which further increases the chemotactic activity in the forming plaques. As leukocytes can release histamine, at this stage a positive feedback loop may be induced resulting in the further recruitment of inflammatory cells.

Similar to the higher number of lymphocytes in plaques of *ApoE*$^{-/-}$ as opposed to *ApoE*$^{-/-}$ *H1R*$^{-/-}$ mice, the blood lymphocyte count was $2.86x10^3 \pm 0.27x10^3$ cells/µl in *ApoE*$^{-/-}$ mice and $1.71x10^3 \pm 0.28x10^3$ cells/µl in *ApoE*$^{-/-}$*H1R*$^{-/-}$ animals ($p<0.01$; Fig. 5C). In contrast, no difference in the number of blood monocytes, neutrophils, basophils, and eosinophils was observed (p=NS; Fig. 5B). The spleen of *ApoE*$^{-/-}$ animals was 40% larger than that of *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice ($p<0.05$;

Fig. 5D); splenic morphology and cellularity were similar (p=NS; data not shown). Size, morphology, and cellularity of inguinal, axillary, and brachial lymph nodes did not differ (p=NS; data not shown).

These differences in blood lymphocyte number and spleen weight between *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice indicate that there is more systemic inflammation in the presence of the H1-receptor and reflect the difference in plaque burden between these groups of mice. The animals had a similar number of blood lymphocytes and a similar spleen weight prior to initiation of the high-cholesterol diet (p=NS; data not shown), underscoring that these differences indeed occur secondary to the development of atherosclerosis.

Summary and Conclusions

This study demonstrates by pharmacological and genetic intervention that histamine enhances the development of atherosclerosis via the H1, but not the H2 receptor. The H1 receptor promotes the formation of atherosclerotic lesions when it is expressed on vascular cells, while its presence on bone marrow derived cells is irrelevant for plaque formation. The effect of the H1 receptor occurs through an increased vascular permeability leading to LDL accumulation and secondary vascular inflammation. These data open interesting perspectives for the prevention and treatment of atherosclerosis.

Materials and Methods

Animal experiments

Animals were kept in individually ventilated cages with free access to food and water, and maintained at 24°C with 12 hours light/dark cycle. All animal experiments were approved by the appropriate authorities.

Pharmacological approach

6-week old *ApoE*$^{-/-}$ male mice were treated for 12 weeks with high cholesterol diet (HCD, Clinton-Cybulski diet, 1.25% cholesterol, Research Diets #D12108). In parallel, the histamine H1 receptor blocker mepyramine (10 mg/kg/day, Sigma) or the H2R blocker ranitidine (100 mg/kg/day, Sigma) was administrated in drinking water.

Genetic approach

Histamine H1 receptor and H2 receptor knockout mice were kindly provided by Prof. T. Watanabe (Graduate School of Medicine, Kyoto University, Yoshida-Konoe machi, Sakyo-ku, Kyoto, Japan). These animals were crossed with *ApoE*$^{-/-}$ mice to generate *ApoE*$^{-/-}$*H1R*$^{-/-}$ and *ApoE*$^{-/-}$*H2R*$^{-/-}$ double knockout mice. 6 week old double knockout male mice and *ApoE*$^{-/-}$ controls were treated with a HCD for 12 weeks.

Plasma cholesterol and triglycerides

Plasma cholesterol was determined using Infinity™ Cholesterol (Thermo Electron Corporation Standard #TR13421) and MC Cal (Abbott #1E65-02); plasma triglycerides were determined using Infinity™ Triglycerides (Thermo Electron Corporation Standard #TR22421) and MC Cal (Abbott #1E65-02). The distribution of lipids within the plasma lipoprotein fractions was assessed by fast-performance liquid chromatography (FPLC) gel filtration using a Superose

6 HR 10/30 column (Pharmacia).

Quantification of atherosclerosis

Animals were sacrificed by cervical dislocation. Blood was collected via a right ventricular puncture into a syringe flushed with heparin (Braun #46613). Aortas were harvested, fixed with 4% paraformaldehyde (PFA, Sigma), and stained with Oil red-O (ORO; Sigma #O9755). AnalysisFIVE was used for quantification of ORO-positive area.

Immunohistochemistry

Specimens were frozen at -80°C in O.C.T. compound (Tissue-Tek #62550-01), cut into 8 µm thick slices, fixed with 4% PFA, and stained with either anti-mouse CD3 (Serotec), CD8 (Santa Cruz), and CD68 (BD) antibodies, followed by incubation with alkaline phosphatase–conjugated secondary antibody (Jackson ImmunoResearch). Percentages of stained area were quantified with Analysis-FIVE program.

RNA expression

Total RNA was isolated using RNeasy Mini Kit (Qiagen #74105). cDNA was generated using Ready-To-Go You-Prime First-Strand Beads (Amersham Bioscience #27-9264-01) and first-strand cDNA primer $pd(N)_6$. Quantitative real-time PCR (qRT PCR) was performed using SybrGreen Jump start kit (Sigma #S-4438). Primers were designed to detect mouse chemokine (C-C motif) ligand 5 (CCL5), histamine H1 receptor, H2 receptor, and histidine decarboxylase. Data were normalized for murine ribosomal S12 and analyzed by the comparative ΔC_t method.

Antibody array

Mouse Atherosclerosis Antibody Array (Tebu-Bio #126AAM-ATH-1L) was performed according to the manufacturer's instructions.

Permeability assay

In vivo permeability assay was performed as described (28). Animals were anesthetized with 80 mg/kg intraperitoneal pentobarbital. Permeability of aorta to LDL was analyzed by using human ^{125}I-LDL. 100 µl of buffer containing 5x10^{7} cpm ^{125} I-LDL and 1% evans blue dye (EBD) was injected retroorbitally 30 minute before aortas were harvested. Radioactivity in ice cold PBS-washed aortas was normalized to blood radioactivity and aorta weight. All measurements were done in triplicate.

FACS

Splenocytes were isolated using a 70 µm nylon mesh and stained with fluorescently labelled anti-mouse CD3 (clone 17A2), CD4 (clone RM4-5), CD8 (clone H35-17.2), CD19 (clone 1D3), and CD44 (clone IM7) antibodies (all from Pharmingen) for 30 minutes at 4°C. Data were collect using DIVAII (BD), and FACS analysis was performed using FlowJo software.

BrdU incorporation

VSMC proliferation *in vivo* was determined by 5'-bromo-2'-deoxyuridine (BrdU) incorporation. Osmotic pumps (Alzet #1007D) containing BrdU (Serva, infusion rate 13 mg/kg/day) were implanted under anaesthesia between the shoulder blades. Incorporation of BrdU was monitored for 7 days after pump implantation in the region of the aortic root (BD Pharmingen #550803). Slides were co-stained using anti-smooth muscle alpha actin antibody conjugated with alkaline phosphatase (Sigma #A5691).

Bone marrow transplantation

BM cells were isolated by flushing femur and tibia bones with ice cold PBS. *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*H1R*$^{-/-}$ recipient mice were lethally irradiated with 1'100 rads (split dose, within 1 hour) and

underwent intravenous rescue injection with 21 x 10^6 BM cells. After 3 weeks of reconstitution time, mice were put on a high cholesterol diet for 12 weeks.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Unpaired Student's t-test was applied for comparison of two groups, ANOVA for three or more groups. For each experiment, $p<0.05$ was considered as statistically significant.

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Legends to Figures and Tables

Figure 1: Histamine H1, but not H2 receptor promotes atherosclerosis

A) *ApoE*$^{-/-}$ mice treated with mepyramine (10 mg/kg/day; n=7; p<0.05), but not ranitidine (100 mg/kg/day; n=8; p=NS) show decreased atherosclerotic plaque formation as compared to mice treated with placebo (control; n=12) after 12 weeks of high cholesterol diet.

B) Representative images (ORO staining) of descending aortas from *ApoE*$^{-/-}$ mice treated with mepyramine, ranitidine, or placebo (control).

C) *ApoE*$^{-/-}$*H1R*$^{-/-}$ (n=9; p<0.01), but not *ApoE*$^{-/-}$*H2R*$^{-/-}$ mice (n=6; p=NS) show decreased atherosclerotic plaque formation as compared to *ApoE*$^{-/-}$ controls (n=13) after 12 weeks of high cholesterol diet.

D) Representative images (ORO staining) of descending aorta from *ApoE*$^{-/-}$*H1R*$^{-/-}$ and *ApoE*$^{-/-}$ *H2R*$^{-/-}$ mice and *ApoE*$^{-/-}$ controls.

Figure 2: Histamine H1 receptor enhances vascular permeability to LDL

A) LDL accumulation is reduced in 18 week old *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice as compared to *ApoE*$^{-/-}$ controls after 12 weeks of high-cholesterol diet (n=5; p<0.05). ^{125}I-LDL in the aortic wall was normalized to blood radioactivity and aortic weight.

B) Representative images (ORO staining) of plaques from *ApoE*$^{-/-}$*H1R*$^{-/-}$ and *ApoE*$^{-/-}$ animals. *ApoE*$^{-/-}$*H1R*$^{-/-}$ animals exhibit less lipid accumulation compared to *ApoE*$^{-/-}$ controls as determined by quantification of ORO-positive area (n=7; p<0.05).

C) LDL accumulation is reduced in 18 week old *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice as compared to *ApoE*$^{-/-}$ controls after 12 weeks of normal diet (n=6-7; p<0.05). ^{125}I-LDL in the aortic wall was normalized to blood radioactivity and aortic weight.

Figure 3: Histamine H1 receptor does not affect vascular smooth muscle cell proliferation

A) No difference in the number of smooth muscle alpha-actin (SMa) – BrdU double positive cells was observed in atherosclerotic plaques of *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice as compared to *ApoE*$^{-/-}$ controls after 8 weeks of high-cholesterol diet (n=7; p=NS).

B) No difference in the SMa positive area was observed in atherosclerotic plaques of *ApoE*$^{-/-}$ *H1R*$^{-/-}$ mice as compared to *ApoE*$^{-/-}$ controls (n=7; p=NS).

C) Immunohistochemical staining for SMa in atherosclerotic plaques of *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice and *ApoE*$^{-/-}$ controls.

Figure 4: Histamine H1 receptor on vascular cells, but not bone marrow-derived cells enhances the development of atherosclerosis

Percentage plaque area (A) and representative en-face ORO stained aortic sections (B) after indicated transplantation protocol. See text for details. **p<0.01, n=4-6.

Figure 5: Enhanced inflammation in the presence of the H1 receptor

A) The number of CD68 (n=9-10; p<0.005), CD3 (n=12-13; p<0.05), and CD4 positive cells (n=10; p<0.05) was higher in atherosclerotic plaques of *ApoE*$^{-/-}$ compared to *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice after 12 weeks of high-cholesterol diet. The number of CD8-positive cells was similar (n=12, p=NS). Three sections of every specimen at a distance of 100 µm each were stained.

B) CCL5 expression at the mRNA and protein level was higher in aorta of *ApoE*$^{-/-}$ compared to *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice after 12 weeks of high-cholesterol diet (n=10; p<0.05).

C) The number of circulating lymphocytes was higher in *ApoE*$^{-/-}$ compared to *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice after 12 weeks of high-cholesterol diet (left panel; n=8-9; p<0.01); in contrast, no difference in the number of monocytes, neutrophils, eosinophils or basophils was observed (table).

D) Spleen weight was higher in *ApoE*$^{-/-}$ compared to *ApoE*$^{-/-}$*H1R*$^{-/-}$ mice after 12 weeks of high-cholesterol diet (left panel; n=10; p<0.05).

Table 1: Plasma triglyceride and plasma cholesterol level, daily food intake, and weight of *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*H1R*$^{-/-}$- mice after 12 weeks of HCD.

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Figure 1

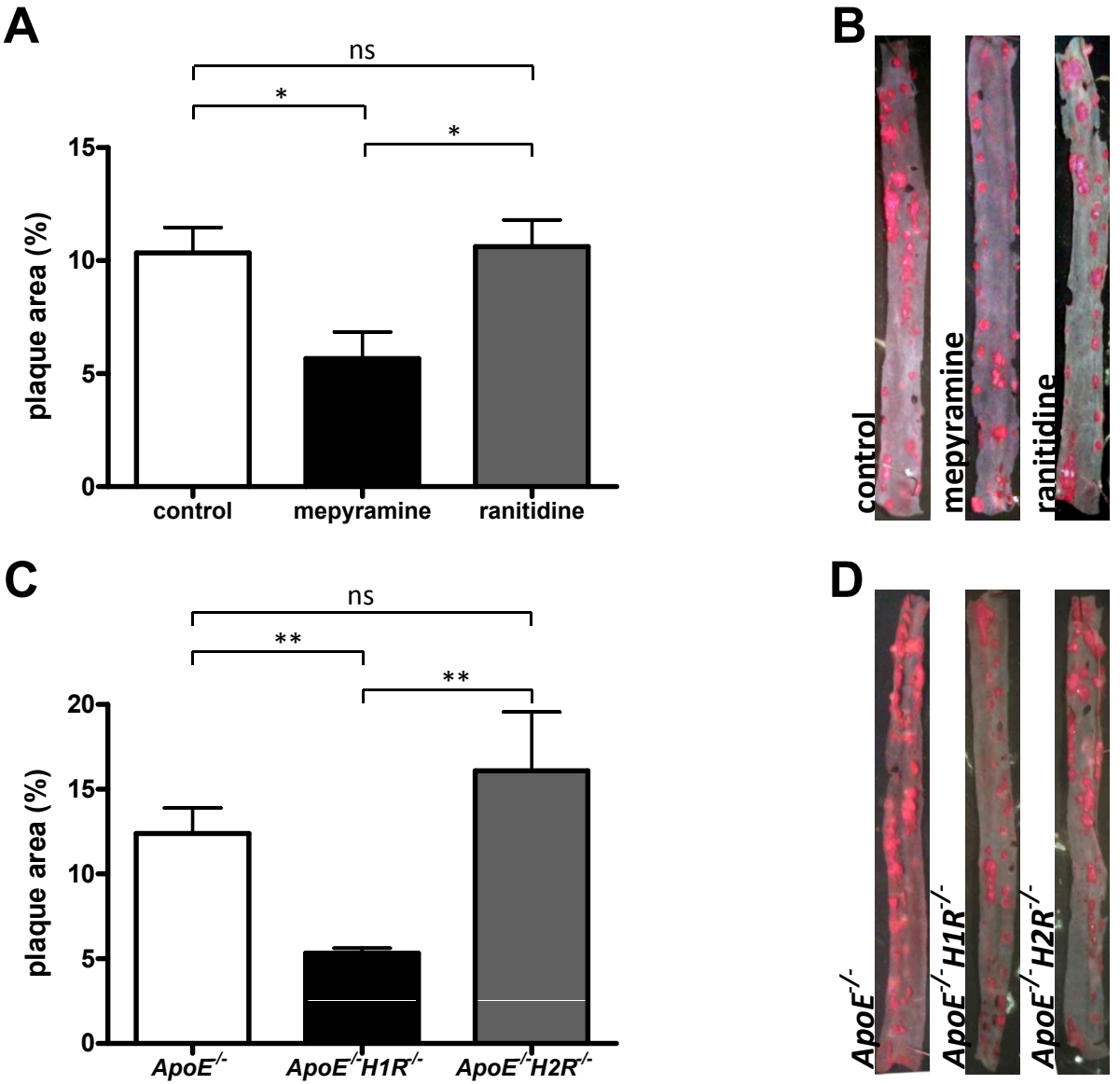

A
ns
*
*
15
10
5
0
plaque area (%)
control
mepyramine
ranitidine
B
control
mepyramine
ranitidine
C
ns
**
**
20
15
10
5
0
plaque area (%)
ApoE-/-
ApoE-/-H1R-/-
ApoE-/-H2R-/-
D
ApoE-/-
ApoE-/-H1R-/-
ApoE-/-H2R-/-

Figure 2

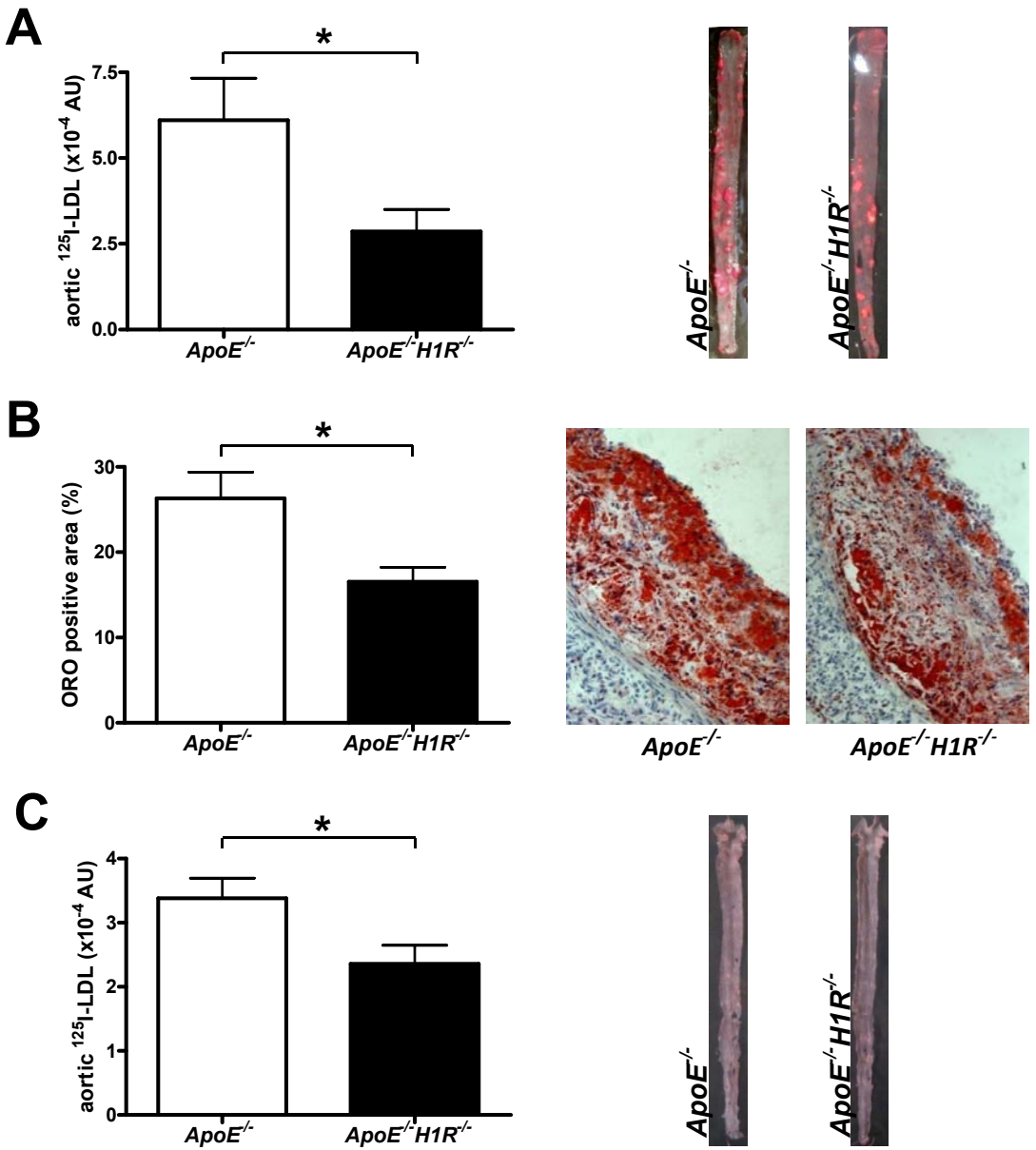

A
*
aortic ^{125}I-LDL (x10^{-4} AU)
7.5
5.0
2.5
0.0
ApoE$^{-/-}$
ApoE$^{-/-}$H1R$^{-/-}$
ApoE$^{-/-}$
ApoE$^{-/-}$H1R$^{-/-}$
B
*
ORO positive area (%)
30
20
10
0
ApoE$^{-/-}$
ApoE$^{-/-}$H1R$^{-/-}$
ApoE$^{-/-}$
ApoE$^{-/-}$H1R$^{-/-}$
C
*
aortic ^{125}I-LDL (x10^{-4} AU)
4
3
2
1
0
ApoE$^{-/-}$
ApoE$^{-/-}$H1R$^{-/-}$
ApoE$^{-/-}$
ApoE$^{-/-}$H1R$^{-/-}$

Figure 3

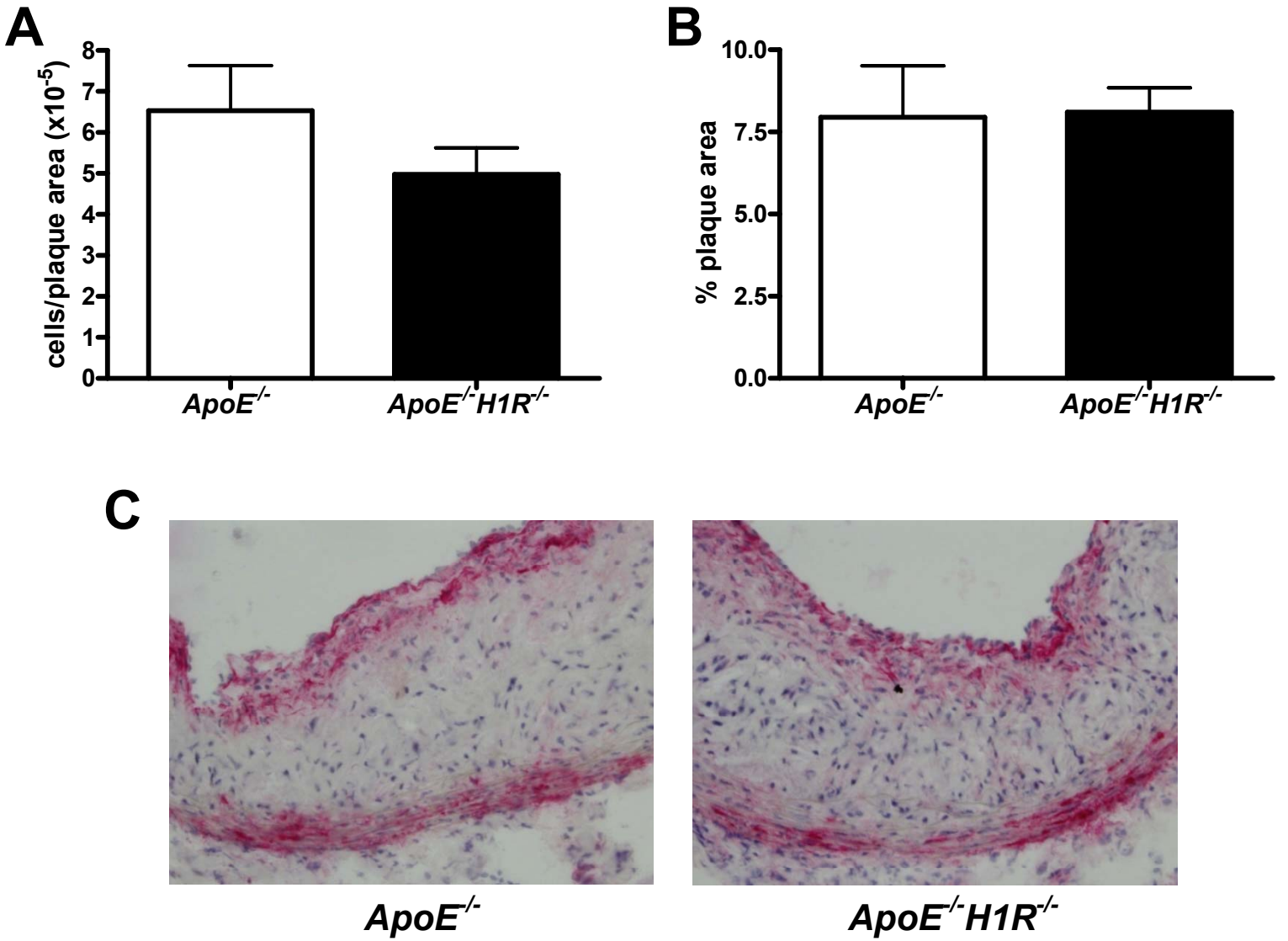
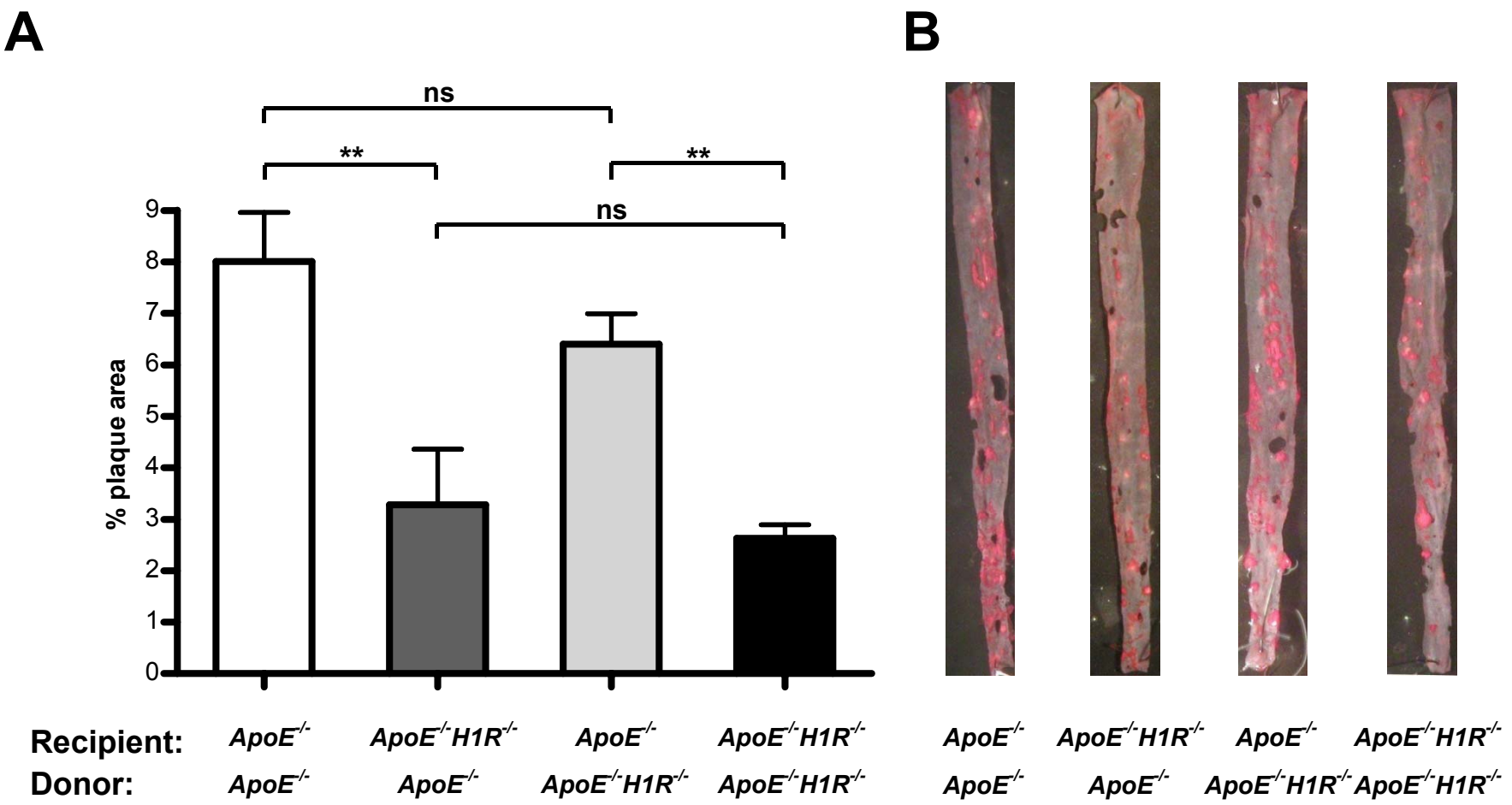

A
cells/plaque area (x10-5)
0
1
2
3
4
5
6
7
8
ApoE-/-
ApoE-/-H1R-/-
B
% plaque area
0.0
2.5
5.0
7.5
10.0
ApoE-/-
ApoE-/-H1R-/-
C
ApoE-/-
ApoE-/-H1R-/-

Figure 4


A
B
ns
**
**
ns
% plaque area
0
1
2
3
4
5
6
7
8
9
Recipient:
Donor:
ApoE-/-
ApoE-/-
ApoE-/-H1R-/-
ApoE-/-
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ApoE-/-H1R-/-
ApoE-/-H1R-/-
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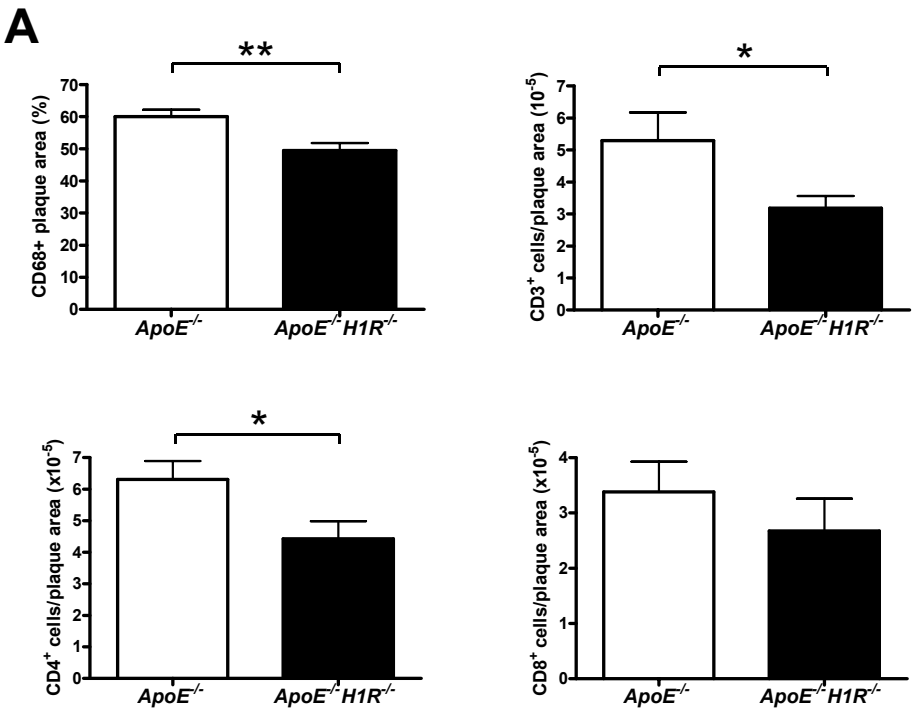
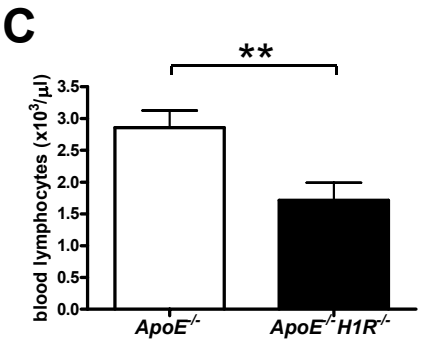
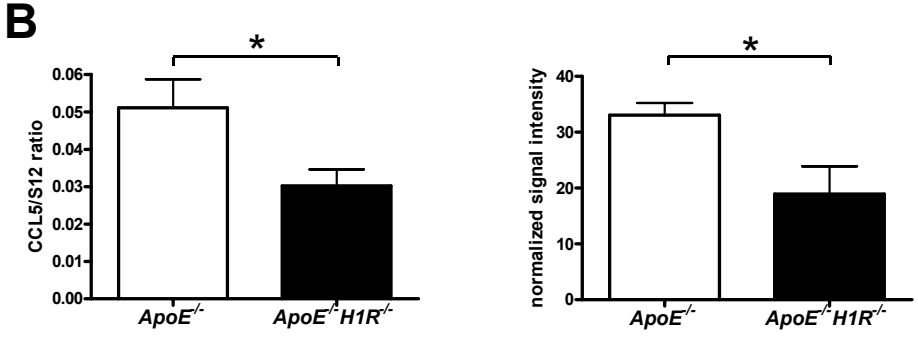
ApoE-/-H1R-/-

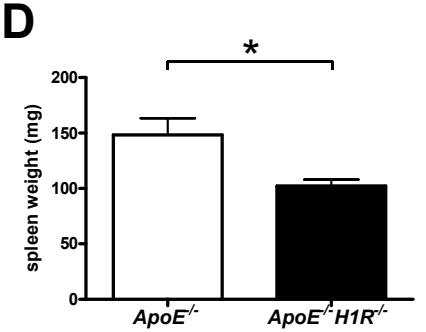
Figure 5





cell type	*ApoE*$^{-/-}$ (10^3/µl)	*ApoE*$^{-/-}$*H1R*$^{-/-}$ (10^3/µl)
monocytes	2.22 ± 0.42	1.74 ± 0.19
neutrophils	0.21 ± 0.05	0.13 ± 0.02
eosinophils	0.23 ± 0.06	0.27 ± 0.06
basophils	0.04 ± 0.06	0.06 ± 0.02





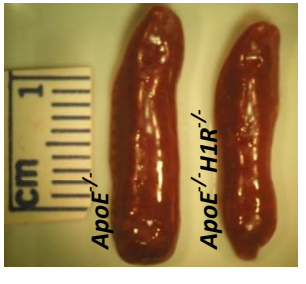


Table 1

	triglyceride (mmol/L)	cholesterol (mmol/L)	daily food intake (g)	weight (g)
***ApoE*$^{-/-}$**	0.61 ± 0.14	17.23 ± 7.21	2.75 ± 0.32	28.38 ± 2.38
***ApoE*$^{-/-}$*H1R*$^{-/-}$**	0.84 ± 0.31	16.24 ± 4.42	2.73 ± 0.40	31.22 ± 3.71*

“Accelerated early atherosclerosis in mice deficient in *L-selectin*”. Manuscript in preparation.

Own contribution:

1) Animal breeding, generation of *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice
2) Animal experiments
    - Harvesting of the mice
    - En-face Oil-red O staining of aortas
    - Quantification of atherosclerosis
3) Immunohistochemistry
    - Cryosection of aortic root, spleen and lymph node
    - Immunostainings (CD3, CD4, CD8; CD68)
    - Analysis of immunostainings (AnalySIS-Five)
4) FACS analysis of murine blood
5) Isolation of mRNA and protein from mouse aorta, spleen and lymph nodes
6) qRT PCR

Accelerated early atherosclerosis in mice deficient in *L-selectin*

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Short title: L-selectin in atherosclerosis

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Abstract

Atherosclerosis is an inflammatory disease characterized by accumulation of leukocytes in the arterial intima. Members of the selectin family of adhesion molecules are important mediators of leukocyte extravasation. It is not known, however, whether L-selectin (L-sel) is involved in the pathogenesis of atherosclerosis.

To address this issue, we crossed *L-sel*$^{-/-}$ animals with mice lacking *Apolipoprotein E* (*ApoE*$^{-/-}$). We analyzed double-knockout *ApoE / L-sel* (*ApoE*$^{-/-}$ *L-sel*$^{-/-}$) mice and the corresponding *ApoE*$^{-/-}$ controls both without and with a high cholesterol diet (HCD).

After 6 weeks of HCD *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice developed on average 2.46% ± 0.54% aortic lesions which is two fold higher than *ApoE*$^{-/-}$ controls (1.13% ± 0.19%). This effect was even more pronounced in 6 month old *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ animals fed a normal diet. In contrast, after 12 weeks of HCD, there was no difference in atheroma formation between the two groups of animals. Leukocyte rolling in atherosclerotic aorta was similar in *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ animals. In line with this, atherosclerotic plaques did not exhibit any alterations in cellular composition upon *L-sel* deletion after 6 and 12 weeks of HCD. Mice lacking *L-sel* exhibited reduced size and cellularity of peripheral lymph nodes, increased size of spleen, and increased number of blood lymphocytes after 6 and 12 weeks of HCD.

These results indicate that L-sel is a negative regulator of early, but not

advanced atherosclerosis. This effect is neither associated with leukocyte capture nor abnormal leukocyte distribution.

Introduction

Endothelial activation followed by leukocyte accumulation is the key event in early atherosclerosis [1]. The selectin family of adhesion molecules mediates initial rolling and tethering of inflammatory cells along activated endothelium [2-6]. It consists of three closely homologous glycoproteins; E-selectin (E-sel), P-selectin (P-sel), and L-selectin (L-sel) that all bind glycoproteins and glycolipids bearing sialyl Lewis X (sLeX) in a calcium-dependent manner [7, 8]. E-sel is expressed on endothelial cells, whereas P-sel on both EC and platelets on which they are expressed on the cell surface following stimulation. L-sel, on the other hand, is expressed constitutively on the majority of leukocytes [6]. It exhibits adhesive as well as signaling functions [9, 10] and is particularly important for lymphocyte homing to secondary lymphoid organs [5, 11]. Indeed, animals lacking *L-sel* display altered size of secondary lymphoid tissues, increased number of blood lymphocytes, and abnormal immunoglobulin profile [11-13]. Moreover, *L-sel* deficient mice show reduced *in vivo* leukocyte rolling along cytokine-stimulated endothelium. This phenomenon is particularly well documented in blood vessels belonging to the microcirculation and depend primarily on a lack of L-sel-mediated interactions between leukocytes that regulate enhanced capture of cells from the free flow [14, 15]. Indeed, functional L-sel ligand activity has not been identified on inflamed EC [16].

Since selectins are known to regulate leukocyte recruitment in inflammation, they are interesting candidates to study in the context of

atherogenesis. Indeed, mice deficient in E- and P-sel display attenuated development of atherosclerosis [2, 17]. Interestingly, it was reported that lymphocyte recruitment into the aortic wall during atherosclerosis development is partially L-sel dependent [18]. However, there are no *in vivo* reports addressing the importance of L-sel in atherosclerosis development *in vivo*. Here, we crossed *L-sel*$^{-/-}$ mice with *Apolipoprotein E*-deficient mice (*ApoE*$^{-/-}$) to investigate the relevance of L-sel on both early and advanced stage of atherosclerosis. Interestingly, we showed that atherosclerotic mice deficient in *L-sel* have an increased early atherosclerosis. These data demonstrate the complexity of the roles for the immune system in atherogenesis.

Results

Deletion of *L-selectin* increases early, but not advanced atherosclerosis.

Development of atherosclerosis was monitored in descending aortas using Oil red-O (ORO) staining. Percentage of aorta occupied by atherosclerotic plaques was two fold higher in 12 week old *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ animals fed a HCD for 6 weeks when compared to age and diet-matched *ApoE*$^{-/-}$ controls (2.46% ± 0.54% versus 1.13% ± 0.19% respectively; P<0.05; Fig. 1A). Effect of *L-sel* deletion was even more pronounced in 6 month old animals fed a normal chow diet. These animals had on average 10.45 ± 2.58% of descending aorta stained by ORO, whereas controls only 1.87 ± 0.37% (P<0.05; Fig. 1B). In contrast 18 week old *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ animals fed a HCD for a time period of 12 weeks exhibited on average similar atherosclerotic burden (11.80 ± 1.86%) as *ApoE*$^{-/-}$ animals (13.89 ± 2.06%; P=ns; Fig. 1C). There was no difference between control mice and double knockouts in plasma cholesterol level in any of the groups (P=ns; Table S1).

Deletion of *L-selectin* does not influence leukocyte capture and rolling in atherosclerosis.

Leukocyte capture and rolling were assessed using intravital microscopy during 30 seconds. Total number of cells rolling along the aortic endothelium was similar in *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ and *ApoE*$^{-/-}$ animals (P=ns; Fig. 2A) and proportional to total leukocyte capture (R^2=0.8; Fig. 2B). There was no difference between *ApoE*$^{-/-}$

and *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ animals in primary leukocyte capture (5.4 ± 1.3 cells versus 5.5 ± 1.3 cells respectively; P=ns; Fig. 2C) and a tendency towards reduced secondary leukocyte capture (1.28 ± 0.92 cells versus 0.17 ± 0.14 cells respectively; P=ns; Fig. 2D).

Deletion of *L-selectin* does not influence leukocyte accumulation into atherosclerotic plaques.

In order to monitor composition of atheroma upon *L-sel* deletion we performed immunostainings of the atherosclerotic plaques in the aortic root area. Macrophages were visualized with anti-CD68 antibody, lymphocytes with anti-CD3 antibody and T-helper cells and T-cytotoxic cells with anti-CD4 and anti-CD8 antibodies respectively. *L-sel* deletion did not result in altered CD68 positive area in either early (71.10% ± 1.36% versus 74.44% ± 2.90%; P=ns; Fig. 3A) or advanced atherosclerosis (48.20% ± 4.13% versus 39.62% ± 1.99%; P=ns; Fig. 3A). There was no difference between *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice in average number of CD3 (P=ns; Fig. 3B), CD4 (P=ns; Fig. 3C), and CD8 positive cells when related to a plaque area (P=ns; Fig. 3D) after 6 and 12 weeks of HCD. Increased duration of HCD resulted in decreased macrophage/plaque area ratio, which did not depend on the genotype of animals (P<0.05; Fig. 3A). There was no diet-dependent difference in accumulation of T-cells (P=ns). The expression of cytokines was similar in aortas of *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice compared to *ApoE*$^{-/-}$ controls after both 6 and 12 weeks of HCD (P=ns; Table 1). In contrast, irrespectively of the genotype, animals after 12 weeks of HCD exhibited

consistently enhanced cytokine production compared to animals after 6 weeks of HCD (P<0.05; Table 1).

Increased number of blood lymphocytes upon *L-selectin* deletion.

We observed 1.4 fold and 1.6 fold increased number of blood lymphocytes in *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ animals compared to *ApoE*$^{-/-}$ controls after 6 and 12 weeks of HCD respectively (P<0.05; Fig. 4A). Indeed total number of CD8^{+} cells and CD19^{+} cells was on average 20% to 40% increased upon L-sel deletion irrespectively of the time of HCD (P<0.05; Fig. 4B and C). After 6 weeks of HCD there was tendency towards increased number of CD4^{+} cells (P=0.24), which became significant after 12 weeks of HCD (P<0.05; Fig. 4D). Increased number of blood lymphocytes in *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice was associated with increased number of naive T helper/CD4^{+}CD44^{-} cells (P<0.05; Fig. 4E) rather than activated T helper/CD4^{+}CD44^{+} cells (P=ns after 6 weeks of HCD, P<0.05 after 12 weeks of HCD; Fig. 4F). We did not detect any significant difference in blood composition of mice after 6 and 12 weeks of HCD in any of the genotypes.

We observed significant reduction in the size (P<0.05; Fig. S1 A) and cellularity of peripheral lymph nodes (P<0.05; Fig. S1 B). In contrast, size of the spleen was on average 30% increased upon *L-sel* deletion in both time-points (P<0.05; Fig; S2 A), this was not associated with altered cellularity of the organ (P=ns; Fig. S2 A) or altered cytokine production (P=ns, data not shown).

Materials and Methods

Mice

L-sel knockout mice backcrossed for 9 generations to C57BL/6 background were a kind gift from Dr Lubor Borsig (Physiology Institute, Zurich University, Switzerland). *L-sel* knockouts were cross-bred with *ApoE*$^{-/-}$ mice to generate *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ and littermate *ApoE*$^{-/-}$ controls. 6 week old males were fed for 6 or 12 weeks a HCD (Clinton-Cybulski diet, 1.25% cholesterol, Research Diets #D12108). In addition 6 month old *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ animals and controls fed a normal chow diet were analyzed. All animal experiments were approved by the appropriate authorities.

Quantification of atherosclerosis development

Under anesthesia, blood was collected through right ventricular puncture. Mice were then perfused with ice-cold saline. Aortas were harvested for RNA isolation, en face, and histological analysis. Thoracic/abdominal part of aortas were fixed overnight with 4% paraformaldehyde (PFA), washed 3 times with ice-cold PBS, and stained for 3 hours with Oil red-O (Sigma #O9755). Quantification of aortic plaque area was performed using AnalysisFIVE software.

Plasma triglyceride and cholesterol level

Plasma cholesterol level was determined using Infinity™ Cholesterol (Thermo Electron Corporation Standard #TR13421) and MC Cal (Abbott #1E65-

02), plasma triglycerides using Infinity™ Triglycerides (Thermo Electron Corporation Standard #TR22421) and MC Cal (Abbott #1E65-02). The distribution of lipids within the plasma lipoprotein fractions was assessed by fast-performance liquid chromatography (FPLC) gel filtration using a Superose 6 HR 10/30 column (Pharmacia).

Immunohistochemistry

Tissue was harvested, mounted in O.C.T. compound (Tissue-Tek #62550-01) and frozen at -20°C. Three to five 8 μm-thick slices were fixed with 4% PFA and stained with anti-mouse CD3, CD4 CD8, or CD68 antibodies (Serotec), followed by incubation with alkaline phosphatase–conjugated secondary antibody (Jackson ImmunoResearch). Percentages of stained area were quantified with AnalySIS-FIVE program.

Intravital microscopy

Mice were anesthetized with isofluran. The aorta was prepared as described previously [19]. Briefly, the abdomen was opened by a midline incision and the intestines were retracted. The peritoneum was then dissected to expose the abdominal aorta. The exposed tissue was superfused with a thermostated (37°C) bicarbonate-buffered saline solution. Microscopic observations were made using an intravital microscope (Leitz Biomed) with a water immersion objective (Leitz SW 25×). Epi-illumination fluorescence microscopy (Leitz Ploem-o-pac, filter block M2 illuminated by a cooled infrared filtered lamp (Osram HBO

200W/4)) was started 2 minutes after labeling of circulating leukocytes with an intravenous injection of rhodamine 6G (0.3 mg/ml, 0.67 mg/kg). Images were televised and recorded on videotape using a VNC-703 video camera. Leukocyte rolling flux was determined as the average number of leukocytes rolling within a 10000 μm^2 area during 30 seconds within a total observation time of at least 180 seconds.

FACS

Blood cells were stained with fluorescently labeled anti-mouse CD4 (PE-conjugated, clone RM4-5), CD8 (PE-Cy7-conjugated, clone H35-17.2), CD19 (PE-Cy7-conjugated, clone 1D3), and CD44 (APC-conjugated, clone IM7) antibodies (Pharmingen) for 30 minutes at 4°C. Erythrocytes were lyzed following staining using commercially available lysis buffer (BD #555899). Data were collected using DIVAII (BD), and FACS analysis was performed using FlowJo software.

Statistical analysis

The results were expressed as mean ± S.E.M. Comparison of means was carried out by Student's t-test or ANOVA in case of multiple comparisons. For each experiment, $P<0.05$ was accepted as statistically significant.

Discussion

Accumulation of leukocytes into the arterial wall is an important pathogenic event in atherosclerotic plaque development. It is well documented that the selectin family of adhesion molecules mediates the initial attachment of leukocytes to activated endothelium, representing the first step of leukocyte emigration into sites of inflammation [2, 6]. Recently, it was postulated that lymphocyte recruitment during atherosclerosis development is partially L-sel dependent [18]. Thus, we hypothesized that deletion of *L-sel* might affect atherogenesis due to impaired leukocyte rolling and capture. To test this hypothesis we generated *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice and compared them to *ApoE*$^{-/-}$ controls.

Despite the partial L-sel dependence of lymphocyte recruitment in atherogenesis, we found that L-sel does not promote atherosclerotic lesion formation in 6 month old *ApoE*$^{-/-}$ mice fed a normal chow diet as well as after a 6 or 12 week HCD. This observation suggests that under conditions of *L-sel* deletion, the presence of other members of the selectin family is sufficient to maintain leukocyte-endothelium interactions. It is consistent with some previous reports on overlapping functions of the selectins [17, 20, 21]. Indeed, any of the selectins can support some leukocyte rolling, and only the deletion of all three family members abolishes leukocyte recruitment [20, 21]. Combined, but not single deletion of E-sel and P-sel results in reduced atherosclerosis development [17]. In line with this, we did not observed any difference between *ApoE*$^{-/-}$ *L-sel*$^{-/-}$

and *ApoE*$^{-/-}$ mice with regard to leukocyte capture or rolling in atherosclerotic aorta.

The effect of *L-sel* deletion on atherosclerotic lesion formation seems to contradict other studies emphasizing the importance of L-sel in the initial stages of leukocyte extravasation [11, 14, 22]. However, most of these studies were conducted in microvessels, whereas atherosclerosis is a disease of large arteries. The wall of microvessels is known to exhibit different properties than that of large arteries, including the expression of distinct L-sel ligands [23]. MadCAM-1, the only well characterized *in vivo* ligand of L-sel is expressed in ECs from the microcirculation, but not in larger blood vessels [24]. Thus, the effect of L-sel on leukocyte rolling in venules or arterioles may not be relevant for atherosclerosis development. In line with this, the presence of a functional L-sel ligand on inflamed endothelium remains controversial [16, 25], and some studies demonstrated that primary capture resulting from leukocyte-endothelium interaction is not affected by L-sel [14]. Thus, we monitored both primary capture as well as secondary capture defined as the interaction of L-sel with selectin glycoprotein ligand-1 (PSGL-1) on the surface of other leukocytes. We did not, however, observe any differences in primary leukocyte capture and only a trend towards decreased secondary capture upon *L-sel* deletion. This observation indicates either that L-sel is not essential for secondary leukocyte capture or secondary capture does not affect leukocyte extravasation during atherosclerosis development.

In the study postulating that leukocyte accumulation into the arterial wall during atherosclerosis development is partially L-sel dependent, accumulation of fluorescently labeled *L-sel* deficient lymphocytes to atherosclerotic aortas was monitored 24 and 72 hours after adaptive transfer to *ApoE*$^{-/-}$ recipients. Upon these conditions deletion of *L-sel* resulted in a 50-57% reduction in leukocyte rolling. In contrast, we applied intravital microscopy to study leukocyte rolling along the aorta of *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice during atherosclerosis development *in vivo*. Adaptively transferred cells do not function in their natural environment and may exhibit altered properties due to the isolation procedure; in contrast, we investigated the intrinsic properties of native leukocytes during atherogenesis. Hence, the discrepancy between these two observations may result from the different experimental setting.

Plaques of *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice exhibited similar numbers of macrophages and T cells as compared to *ApoE*$^{-/-}$ controls. Thus, the cellular composition of atherosclerotic plaques was not affected by *L-sel* deletion irrespective of the presence or the duration of HCD. This supports the interpretation that the absence of *L-sel* is not sufficient to influence the initial stages of leukocyte transmigration during atherosclerosis development. In line with this, we did not detect any difference in cytokine production in atherosclerotic vessel walls, indicating a similar extent of the local inflammatory state in *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ animals. In contrast, animals treated for 12 weeks with a HCD exhibited enhanced aortic cytokine levels as compared to animals treated 6 weeks with a HCD, consistent with a more advanced stage of atherosclerosis in these mice.

L-sel had an atheroprotective effect in animals without a HCD and in those fed a HCD for 6 weeks, but not for 12 weeks. This indicates a role for L-sel in the early, but not the advanced stages of atherosclerosis. The increased atherosclerotic burden in the animals lacking *L-sel* may result from an altered local inflammation or abnormal systemic leukocyte distribution.

The inflammatory state of atherosclerotic plaques is an important determinant of atherosclerosis development [1]. Indeed, it was demonstrated that deletion of cytokines and adhesion molecules expressed specifically by the vascular wall attenuates atherosclerosis development [17, 26, 27]. We did not, however, observe any difference between *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice and controls in aortic levels of pro- and anti-inflammatory molecules, suggesting a similar inflammatory state within the plaques in these animals. In line with this, there was no difference between *L-sel* deficient mice and controls in cellular composition of the atherosclerotic plaques.

Atherosclerosis affects not only the wall of blood vessels, but also provokes changes at the systemic level [28, 29]. Deletion of *L-sel* resulted in abnormal systemic leukocyte distribution, which could potentially affect atherosclerosis development [30]. As expected, both size and cellularity of peripheral lymph nodes were decreased in *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice as compared to *ApoE*$^{-/-}$ controls. This is consistent with observation that migration of naive lymphocytes into peripheral lymph nodes is practically eliminated upon *L-sel* deletion [11, 13]. The spleen cannot fully compensate for the cessation of leukocyte homing to peripheral lymph nodes [11], thus, *L-sel* deficiency result in

increased numbers of blood lymphocytes. Although changes in blood leukocyte numbers were demonstrated to correlate with progression of atherosclerosis [31], leukocyte capture and cellular plaque composition were not affected by the absence of *L-sel*. Moreover, leukocyte distribution in *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice was comparable in animals fed 6 and 12 weeks a HCD. Hence, they do not explain the differences in atherosclerotic plaque formation between these animals.

To summarize, we demonstrated for the first time that L-sel attenuates early stages of atherosclerosis, which is not associated with reduced leukocyte rolling or leukocyte accumulation into atherosclerotic plaques. Further studies are required to clarify the mechanism of this phenomenon.

Legends to Figures and Tables

Figure 1

Atherosclerosis development in *L-sel*$^{-/-}$ mice lacking *ApoE*. Representative pictures of aortas stained with Oil-Red-O (ORO). Atherosclerotic lesion area expressed as a percentage of total aortic area. Increased atherosclerotic burden in A) 12 week old *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ animals after 6 weeks of HCD (n=11-15; *P<0.05); and B) 6 month old *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ animals fed a normal chow diet as compared to *ApoE*$^{-/-}$ controls (n=5-9; *P<0.05), but not C) 18 week old animals after 12 weeks of HCD (n=10-15; P=ns) as compared to *ApoE*$^{-/-}$ controls.

Figure 2

L-sel does not affect leukocyte capture and rolling during atherosclerosis. A) Leukocyte rolling in atherosclerotic aorta was not affected by the absence of *L-sel* (n=7; P=ns). B) Total capture of leukocytes was proportional to leukocyte rolling flux (R=0.8). There was no difference between *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ animals in either C) primary or D) secondary leukocyte capture (n=7; P=ns).

Figure 3

Similar cellular composition of atherosclerotic plaques in *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ mice and *ApoE*$^{-/-}$ controls. Immunostainings were performed to visualize A) macrophages/CD68^{+}, B) T-lymphocytes/CD3^{+}, C) T helper cells/CD4^{+}, and D) T-cytotoxic cells/CD8^{+} after 6 and 12 weeks of HCD (n=5-13; P=ns).

Figure 4

Altered blood composition in atherosclerotic *L-sel*$^{-/-}$ animals. Increased number of A) total blood lymphocytes (n=5-8; *P<0.05) upon *L-sel* deletion associated with increased number of B) T-cytotoxic cells/CD8^{+} (n=6-7; **P<0.01, $^{\#}$P<0.05). C) B lymphocytes/CD19^{+} (n=5-7; *P<0.05, $^{\#}$P<0.05), D) total/CD4^{+}, and E) naive T-helper cells/CD4^{+}CD44^{-} (n=5-7; *P<0.05, $^{\#\#}$P<0.0001) after 6 and 12 weeks of HCD respectively. F) Number of activated T-cells/CD4^{+}CD44^{+} remained unchanged after 6 weeks of HCD (n=5-7; P=ns) and decreased after 12 weeks of HCD (n=5-7; *P<0.05) compared to corresponding controls.

Table 1

Cytokine mRNA abundance is not affected by an absence of *L-sel*.

Supplement

Figure S1

Decreased A) size (n=5-6; **P<0.01, $^{\#\#}$P<0.01) and B) cellularity (n=5-6; *P<0.05, $^{\#}$P<0.05) of peripheral lymph nodes (LN) in mice lacking *L-sel* after both 6 and 12 weeks of HCD.

Figure S2

A) Increased size of the spleen upon *L-sel* deletion (n=6; **P<0.01, $^{\#}$P<0.05). B) Cellularity of the organ was not affected by *L-sel* deletion (n=5-6; *P>0.05).

Table S1

Plasma cholesterol and triglyceride levels (n=5-12; P=ns).

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Figure 1

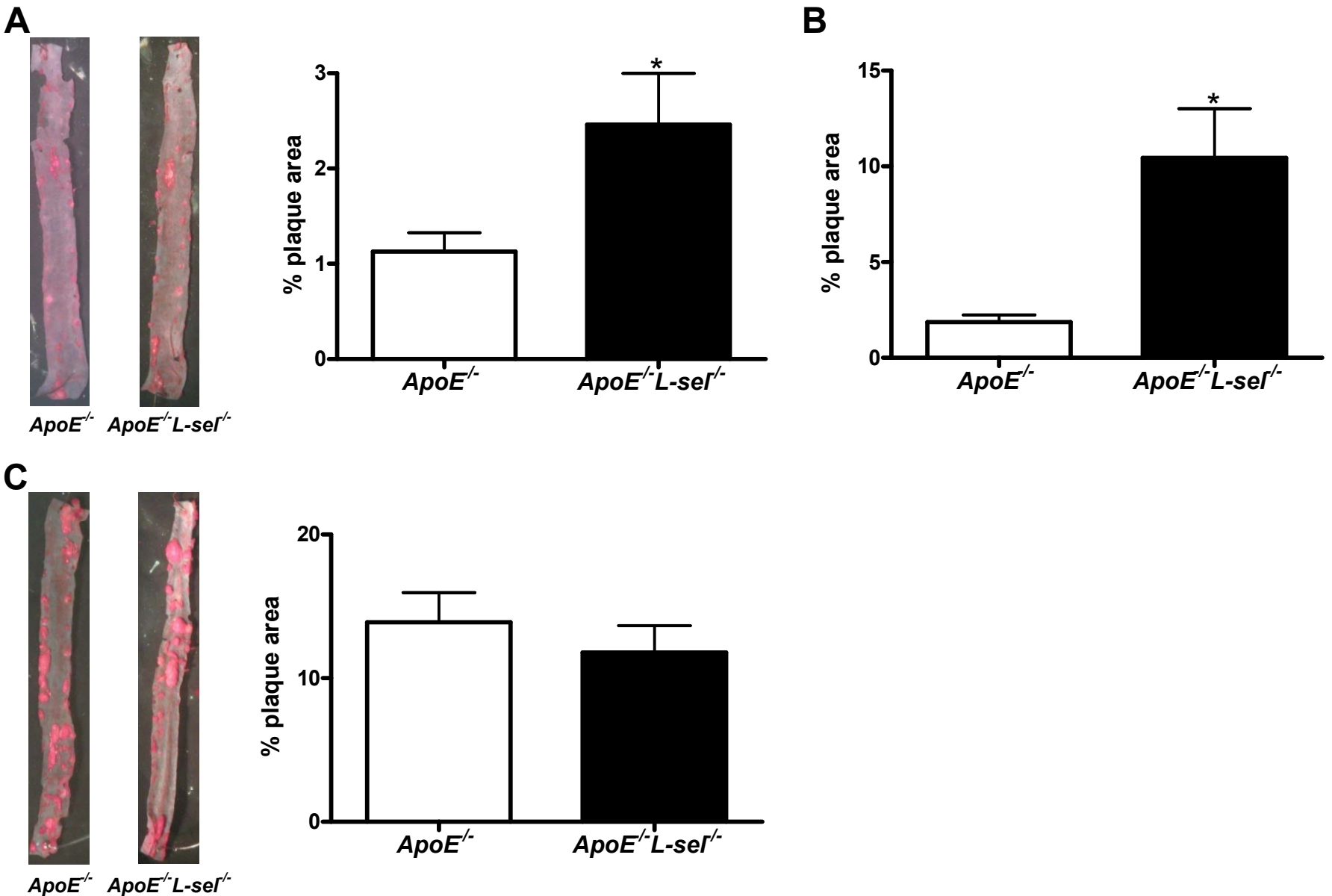
A
ApoE-/-
ApoE-/-L-sel-/-
% plaque area
3
2
1
0
*
ApoE-/-
ApoE-/-L-sel-/-
B
% plaque area
15
10
5
0
*
ApoE-/-
ApoE-/-L-sel-/-
C
ApoE-/-
ApoE-/-L-sel-/-
% plaque area
20
10
0
ApoE-/-
ApoE-/-L-sel-/-

Figure 2

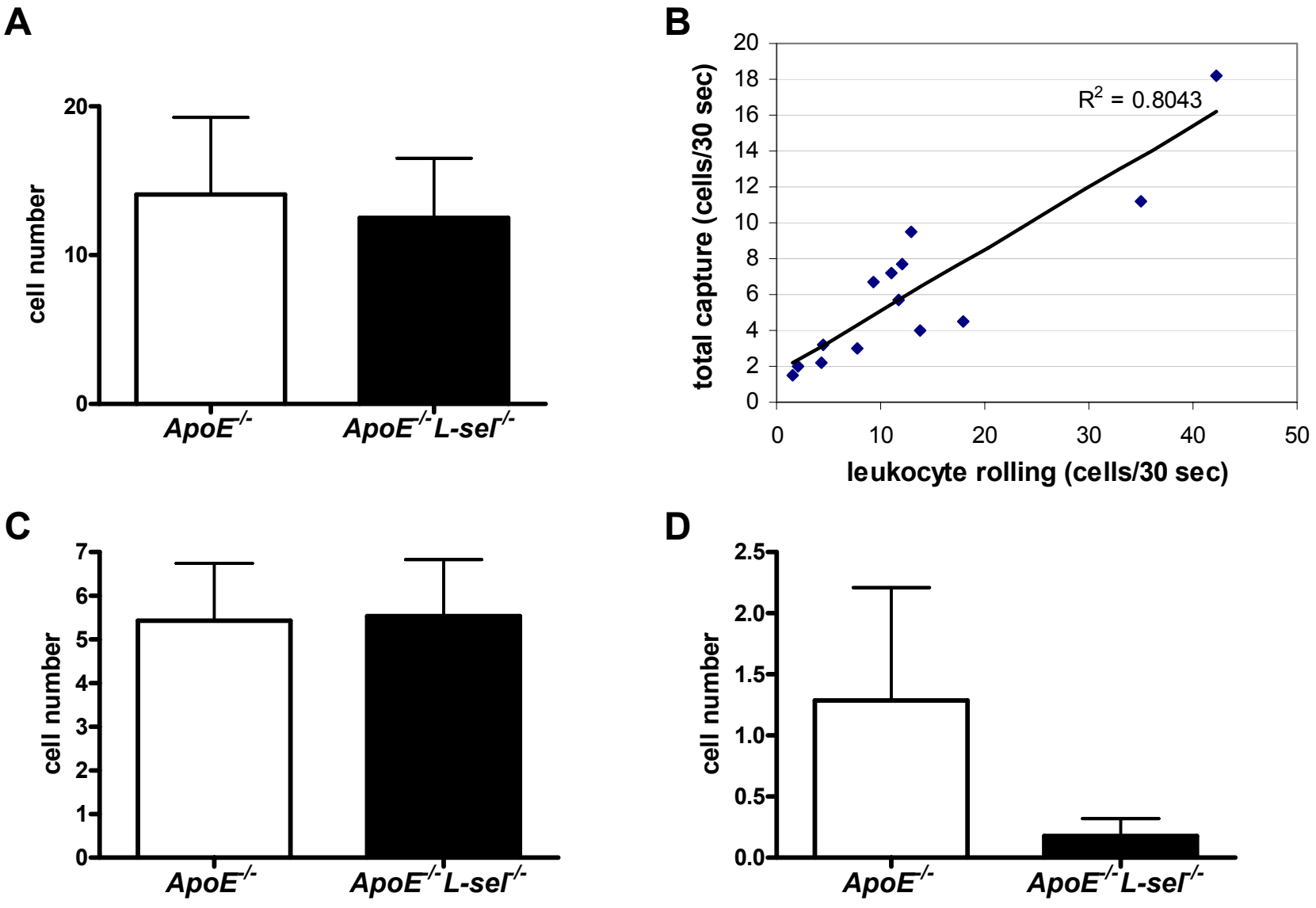
A
cell number
20
10
0
ApoE-/-
ApoE-/-L-sel-/-
B
total capture (cells/30 sec)
leukocyte rolling (cells/30 sec)
R2 = 0.8043
0
2
4
6
8
10
12
14
16
18
20
0
10
20
30
40
50
C
cell number
0
1
2
3
4
5
6
7
ApoE-/-
ApoE-/-L-sel-/-
D
cell number
0.0
0.5
1.0
1.5
2.0
2.5
ApoE-/-
ApoE-/-L-sel-/-

Figure 3

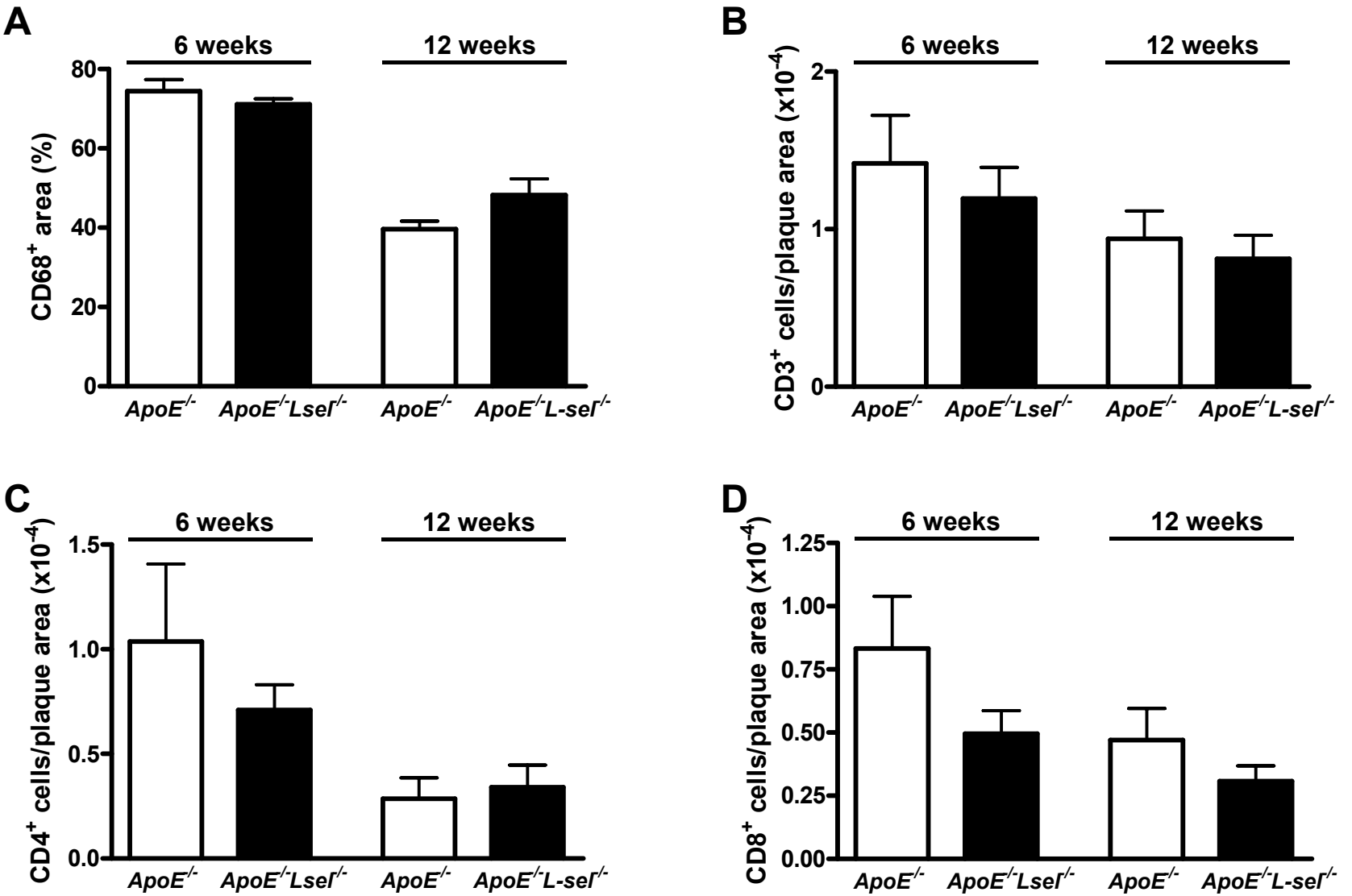
A
6 weeks
12 weeks
CD68+ area (%)
80
60
40
20
0
ApoE-/-
ApoE-/-Lsel-/-
ApoE-/-
ApoE-/-L-sel-/-
B
6 weeks
12 weeks
CD3+ cells/plaque area (x10-4)
2
1
0
ApoE-/-
ApoE-/-Lsel-/-
ApoE-/-
ApoE-/-L-sel-/-
C
6 weeks
12 weeks
CD4+ cells/plaque area (x10-4)
1.5
1.0
0.5
0.0
ApoE-/-
ApoE-/-Lsel-/-
ApoE-/-
ApoE-/-L-sel-/-
D
6 weeks
12 weeks
CD8+ cells/plaque area (x10-4)
1.25
1.00
0.75
0.50
0.25
0.00
ApoE-/-
ApoE-/-Lsel-/-
ApoE-/-
ApoE-/-L-sel-/-

Figure 4

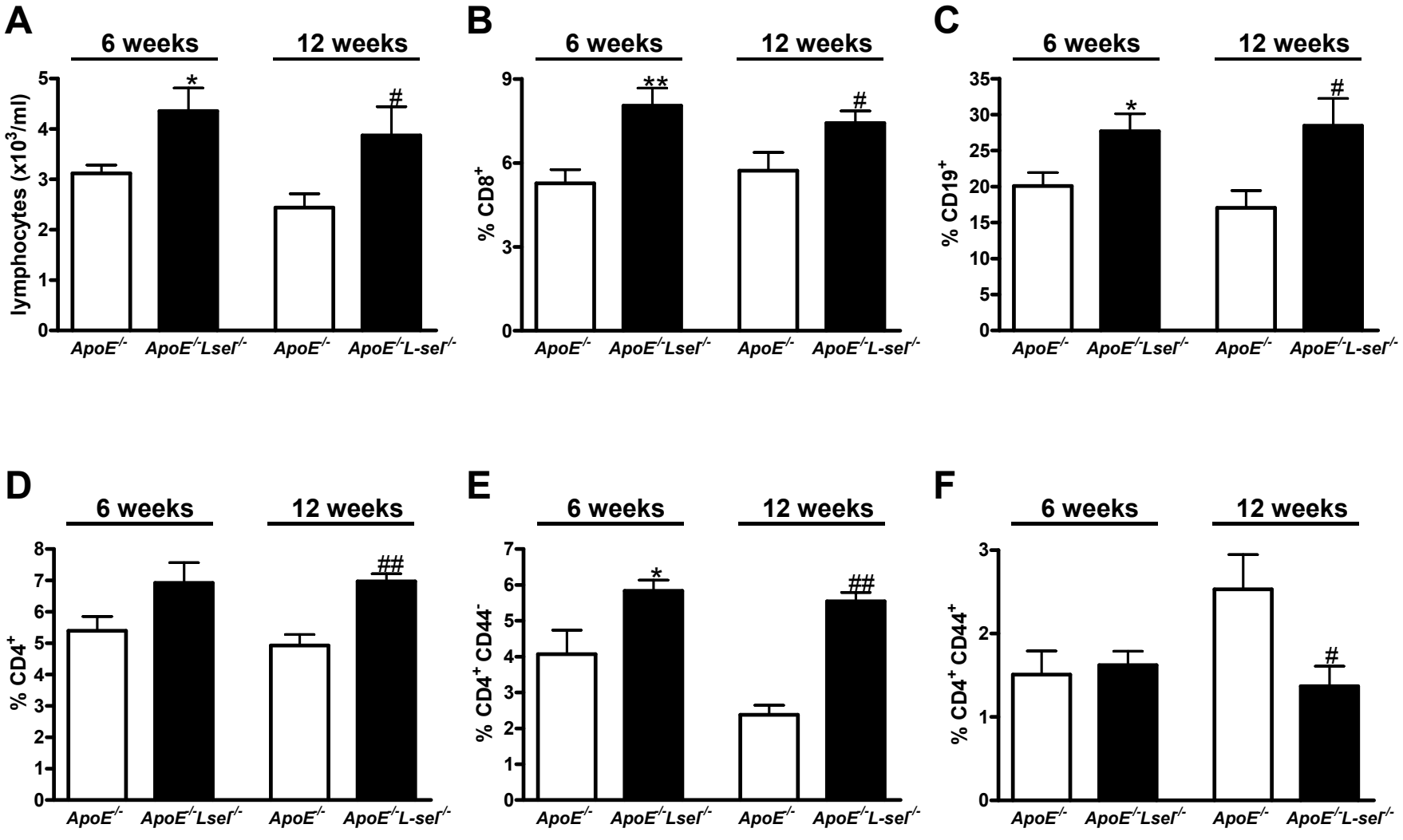
A
6 weeks
12 weeks
lymphocytes (x10^3/ml)
*
#
B
6 weeks
12 weeks
% CD8+
**
#
C
6 weeks
12 weeks
% CD19+
*
#
D
6 weeks
12 weeks
% CD4+
##
E
6 weeks
12 weeks
% CD4+ CD44-
*
##
F
6 weeks
12 weeks
% CD4+ CD44+
#
ApoE-/-
ApoE-/-Lsel-/-
ApoE-/-
ApoE-/-L-sel-/-

Table 1

	6 weeks		12 weeks	
expression level	*ApoE*$^{-/-}$	*ApoE*$^{-/-}$ *L-sel*$^{-/-}$	*ApoE*$^{-/-}$	*ApoE*$^{-/-}$ *L-sel*$^{-/-}$
MCP-1 (x10^{-2})	2.02 ± 0.58	2.59 ± 0.37	5.22 ± 1.47	5.95 ± 1.20
TNFα (x10^{-3})	1.40 ± 0.39	1.40± 0.15	3.90 ± 1.30	4.2 ± 1.01
INFγ (x10^{-4})	4.51 ± 1.8	3.88 ± 0.7	18.71 ± 8.00	25.45 ± 10.30
Mip-1 (x10^{-2})	3.44 ± 0.59	2.84 ± 0.36	5.88 ± 1.88	3.14 ± 0.65
IL-4 (x10^{-4})	4.43 ± 1.20	3.51 ± 0.4	11.01 ± 3.7	10.11 ± 2.7
IL-6 (x10^{-3})	1.11 ± 0.22	1.34 ± 0.25	2.32 ± 0.64	2.22 ± 0.59
IL-10 (x10^{-4})	2.22 ± 1.00	2.79 ± 0.40	6.51 ± 1.60	4.79 ± 1.3

Figure S1

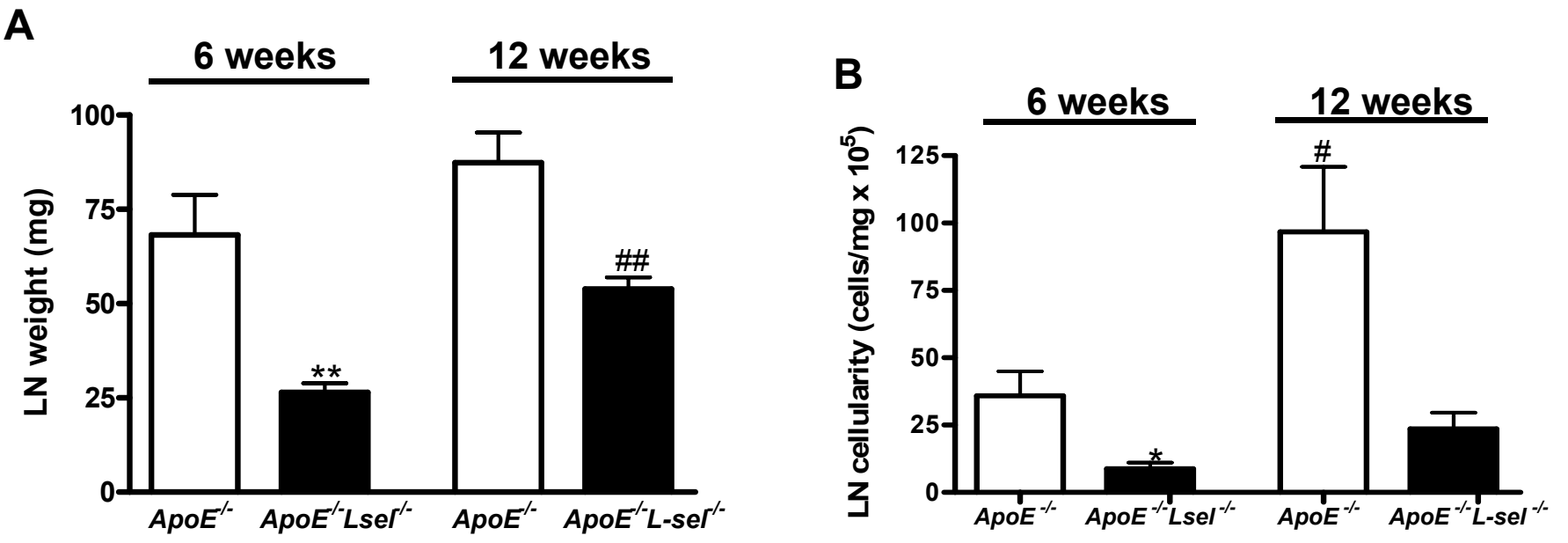
A
6 weeks
12 weeks
LN weight (mg)
100
75
50
25
0
**
##
ApoE-/-
ApoE-/-Lsel-/-
ApoE-/-
ApoE-/-L-sel-/-
B
6 weeks
12 weeks
LN cellularity (cells/mg x 10^5)
125
100
75
50
25
0
*
#
ApoE -/-
ApoE -/-Lsel -/-
ApoE -/-
ApoE -/-L-sel -/-

Figure S2

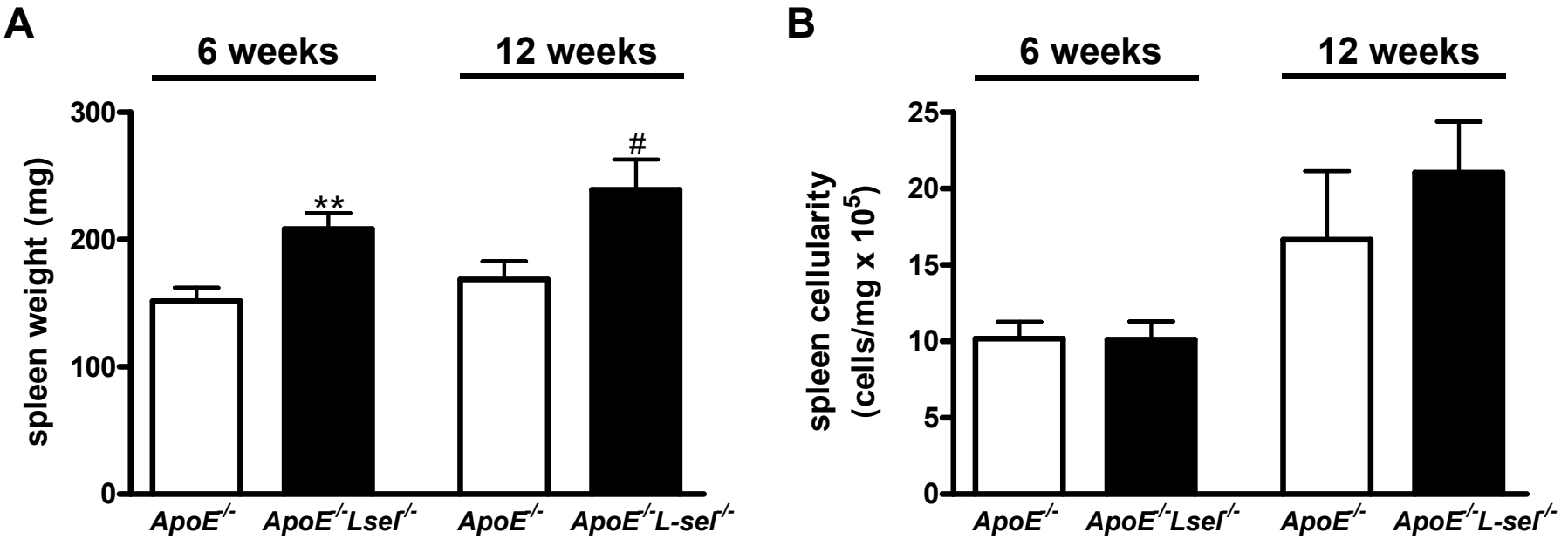
A
6 weeks
12 weeks
spleen weight (mg)
300
200
100
0
**
#
ApoE-/-
ApoE-/-Lsel-/-
ApoE-/-
ApoE-/-L-sel-/-
B
6 weeks
12 weeks
spleen cellularity
(cells/mg x 10^5)
25
20
15
10
5
0
ApoE-/-
ApoE-/-Lsel-/-
ApoE-/-
ApoE-/-L-sel-/-

Table S1

	no		6 weeks		12 weeks	
mmol/L	*ApoE*$^{-/-}$	*ApoE*$^{-/-}$*L-sel*$^{-/-}$	*ApoE*$^{-/-}$	*ApoE*$^{-/-}$*L-sel*$^{-/-}$	*ApoE*$^{-/-}$	*ApoE*$^{-/-}$*L-sel*$^{-/-}$
cholesterol	9.16 ± 1.19	10.48 ± 0.77	32.88 ± 1.53	30.10 ± 1.95	32.31 ± 2.03	30.78 ± 2.53
triglyceride	1.56 ± 0.13	1.73 ± 0.18	1.25 ± 0.19	1.35 ± 0.23	1.27 ± 0.17	0.94 ± 0.08

“Endothelial overexpression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) increases LDL uptake and plaque formation”. Manuscript in preparation.

Own contribution:

1) Animal experiments
    - Harvesting of the mice
    - En-face Oil-red O staining of aortas
    - Quantification of atherosclerosis development
2) Cryosection of aortic roots, preparation for immunohistochemistry
3) Analysis of immunostainings (together with Dr. Alexander Akhmedov)
4) Isolation of cells
    - Isolation of murine ECs
    - Isolation of murine macrophages
5) Cell culture
    - oxLDL uptake by murine EC, FACS analysis (together with Pavani Mocharla)
    - oxLDL uptake by murine macrophages, FACS analysis
    - Preparation of cells for protein and mRNA isolation

Endothelial overexpression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) increases LDL uptake and plaque formation

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Short title: Endothelial LOX-1 overexpression increases atherosclerosis

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Abstract

Oxidized low-density lipoprotein (oxLDL) plays a major role in atherogenesis. Although lectin-like oxLDL receptor-1 (LOX-1) is the main oxLDL receptor expressed by endothelial cells (ECs), its importance in atherogenesis *in vivo* remains unknown.

To address this issue, we generated endothelial-specific *LOX-1* transgenic mice (*LOX-1 TG*) using the Tie2 promoter. Indeed, overexpression of LOX-1 in ECs, but not in macrophages, resulted in enhanced oxLDL uptake. To investigate potential atherogenic effects of LOX-1 *in vivo*, 6-week-old *LOX-1 TG* and wild-type (WT) mice were fed a high cholesterol diet (HCD) for 30 weeks. In addition, *LOX-1 TG* animals were crossbred with *apolipoprotein E* deficient mice ($ApoE^{-/-}$) and fed HCD for 20 weeks. LOX-1 overexpression resulted in enhanced atherosclerosis, which was associated with impaired EC-dependent relaxation to acetylcholine. In addition, in both *LOX-1 TG* and *LOX-1 TG/*$ApoE^{-/-}$ mice, we observed increased aortic expression of adhesion molecules. In line with this, the number of leukocytes in atherosclerotic plaques of animals overexpressing LOX-1 was increased compared to controls.

Thus, endothelial-specific overexpression of LOX-1 leads to increased atherosclerotic burden due to enhanced endothelial cholesterol uptake, increased expression of adhesion molecules, and, as a result, increased leukocyte extravasation. Therefore, *LOX-1* may be a promising therapeutic target for atherosclerosis.

Introduction

Atherosclerosis is a chronic inflammatory disease initiated by endothelial activation and induced, among the others, by the oxLDL uptake [1, 2]. This process leads eventually to adhesion and transmigration of leukocytes into arterial intima and promotes atherosclerotic plaque formation [1, 3]. OxLDL can be scavenged by both, ECs and macrophages [4, 5]. While macrophages internalize oxLDL by receptors such as scavenger receptor A (SR-A), SR-BI, and CD36 [6-8], in ECs, the oxLDL uptake depends primarily on the LOX-1 [9-11].

LOX-1 is a type II membrane glycoprotein with a molecular weight of 50 kDa. It has a C-terminal extracellular C-type lectin-like domain, which is essential for binding to oxLDL [12]. In cultured endothelial cells, activation of LOX-1 by oxLDL upregulates the expression of several genes involved in endothelial dysfunction, such as MCP-1, ICAM-1, and VCAM-1 and reduces NO release [13-15]. Thus, internalization of oxLDL leads to ECs activation and, eventually, atherosclerosis development [13, 16-18]. Indeed, oxLDL-induced endothelial dysfunction has been implicated in atherogenesis and *LOX-1* expression is increased in atherosclerotic plaques [9, 19, 20]. Moreover, it was recently reported that deletion of *LOX-1* attenuates atherosclerosis in *low-density lipoprotein receptor* knockout mice due to reduced collagen deposition and metalloproteinases expression [21].

Although the role of LOX-1 in ECs activation is well described, there are no *in vivo* studies clarifying its relevance in atherosclerosis. Therefore, we generated

LOX-1 TG mice to investigate the importance of endothelial oxLDL uptake in atherosclerotic plaques development *in vivo*.

Results and Discussion

To investigate the role of LOX-1 in atherogenesis, we generated mice with endothelial overexpression of LOX-1 driven by the endothelial-specific *Tie2* promoter [22]. Animals overexpressing LOX-1 in ECs (Fig. 1A) carried a different copy number of *LOX-1* transgene (Fig. 1B). The line 5048.1, which showed the highest transgene expression, was selected for further experiments. Real time PCR analysis revealed highest LOX-1 expression in the aorta, although low expression was also detected in the kidney (Fig. 1C). Aortic LOX-1 overexpression was confirmed in *LOX-1 TG* mice by Western blotting (Fig. 1D). Immunohistochemical staining visually confirmed that specific overexpression of LOX-1 is confined to ECs (Fig. 1E). To further test specificity of LOX-1 overexpression, ECs were isolated from murine aortas. The purity of cells was confirmed with FACS (Fig. 2A). As expected, in cultured ECs isolated from *LOX-1 TG* mice, oxLDL uptake was increased compared to those isolated from WT mice (Fig. 2B, and C). Importantly, there was no difference in Dil-oxLDL uptake in cultured *LOX-1 TG* and WT macrophages (data not shown). Thus, although *Tie2* promoter was reported to be active also in macrophages [23, 24], it appears, that other scavenger receptors like SR-A, CD36, and SR-BI play a dominant role in oxLDL uptake in these cells. These findings underline the specific functional effects of endothelial-specific LOX-1 overexpression.

To examine the effects of LOX-1 overexpression on atherogenesis, we fed *LOX-1 TG* and WT male mice a HCD for 30 weeks starting at 6 weeks of age.

LOX-1 TG mice exhibited increased fatty streak formation in aortic root as compared to WT controls (Fig. 3A, and B). Since plasma lipid levels were comparable in *LOX-1 TG* and WT mice (Tab. 1), this difference is likely to be related to an increased activity of endothelial LOX-1 receptor.

In cultured ECs, LOX-1 activation enhances the expression of chemokines and adhesion molecules [14, 15]. These cellular events, together with the chemoattractant properties of LOX-1 itself [25], could facilitate accumulation of oxLDL as well as macrophage infiltration into the vascular wall leading to progression of atherosclerosis. In line with this, aortic *vascular cell adhesion molecule-1* (*VCAM-1*) and *E-selectin* (*E-sel*) mRNA level was enhanced in the *LOX-1 TG* compared to WT mice (Fig. 4A and B). This was confirmed by immunohistochemistry showing increased endothelial expression of VCAM-1 in *LOX-1 TG* mice (Fig. 4C and D). E-sel expression was not detectable with this method. Adhesion molecules play an important role in the transmigration of leukocytes into atherosclerotic plaques [26]. Indeed, the number of activated macrophages (CD68 positive area) was increased in the aorta of *LOX-1 TG* mice compared to controls (Fig. 4E and F).

To investigate the effects of LOX-1 overexpression on advanced atherosclerosis, *LOX-1 TG* mice were cross-bred with *ApoE*$^{-/-}$ on C57BL/6 background and resulting *LOX-1 TG/ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ littermates were fed a HCD for 20 weeks starting at 6 weeks of age. As an endothelial dysfunction precedes plaque formation [27, 28], we assessed endothelium-dependent relaxation in the aortic rings from both groups. Indeed, in *LOX-1 TG/ApoE*$^{-/-}$ mice,

acetylcholine induced endothelium-dependent relaxation was reduced (pEC50 (-log M): 6.34±0.12); E_{max} (% of contraction) 6.52±3.05%) compared to those of *ApoE*$^{-/-}$ mice (6.67±0.40, P>0.05; E_{max} (% of contraction) 22.18±6.68%; P<0.05). In contrast, endothelium-independent relaxation to sodium nitroprusside was comparable in both groups (data not shown). These data demonstrate profound endothelial dysfunction induced by overexpression of LOX-1 during atherosclerosis development.

Furthermore, atherosclerotic plaque area was increased almost two-fold in *LOX-1 TG/ApoE*$^{-/-}$ as compared to *ApoE*$^{-/-}$ controls (Fig. 5A and B). To determine the effects of endothelial-specific LOX-1 on the expression of adhesion molecules in the *ApoE*$^{-/-}$ background, immunohistochemical staining for VCAM-1 and P-selectin (P-sel) was performed on cross sections of aortic roots (Fig. 6A and D). Analysis revealed increased expression of both VCAM-1 (Fig. 6A and B) and P-sel (Fig. 6D and E) in *LOX-1 TG/ApoE*$^{-/-}$ mice compare to controls. RNA analysis confirmed increased *VCAM-1*, but not *P-sel* abundance (Fig. 6C). It agrees with previous reports demonstrating that expression of P-sel is regulated primarily on the posttranscriptional level [29]. In line with these findings, the number of macrophages (CD68-positive areas; Fig. 7A and B) and T-cells (CD3-positive areas; Fig. 7C and D) was increased in atherosclerotic plaques of *LOX-1 TG/ApoE*$^{-/-}$ mice compared to controls. Thus, LOX-1 overexpression *in vivo* results in increased atherosclerotic lesion formation independent of the plasma lipoprotein levels. Rather, increased endothelial uptake mediated by the overexpression of a functional LOX-1 transgene is involved in that process. Our data correlate well with

a recent study in *LOX-1* knockout mice, where decreased level of atherosclerosis was measured [21]. In other study overexpression of bovine LOX-1 driven by a preproendothelin-1 promoter resulted in inflammatory intramyocardial vasculopathy [30]. However, a preproendothelin-1 promoter, in contrast to the Tie2 promoter used in this study, drives the expression of the LOX-1 transgene predominantly in microvessels leading to a vasculatis rather than to atherosclerotic changes. Furthermore, species differences in the bovine versus murine *LOX-1* gene could bias the results.

In summary, our data strongly support the thesis that endothelial-specific overexpression of LOX-1 leads to endothelial dysfunction and enhanced atherosclerotic plaque formation in both C57BL/6 and *ApoE*$^{-/-}$ mice.

Indeed, we showed increased *in vivo* expression of VCAM-1 and E-sel in aortas of *LOX-1 TG* mice as well as an increased expression of VCAM-1 and P-sel in aortas of *LOX-1 TG/ApoE*$^{-/-}$ mice compared to corresponding controls. This was associated with impaired EC-specific relaxation to acetylcholine. This finding indicates that LOX-1 promotes ECs activation under hyperlipidemic conditions. In line with these molecular events, an increased numbers of leukocytes were observed in the atherosclerotic lesions of mice overexpressing LOX-1.

We believe that the development of strategies aimed at endothelial-specific inhibition of LOX-1 may open new therapeutic approaches for the prevention as well as for the treatment of atherosclerosis.

Materials and Methods

Generation of LOX-1 transgenic mice

To obtain transgenic mice specifically overexpressing *LOX-1* in EC, a murine tyrosine kinase receptor Tie2 promoter was used (Figure 1A). Targeted *LOX-1* gene expression in ECs was achieved using the expression vector pSP14/15, which contains the murine 2 kb Tie2 promoter together with a 10 kb Tie2 enhancer originated from intron 1 of the endogenous murine Tie2 gene (the pSP14/15 vector was a kind gift of Thomas N. Sato, MD, PhD, University of Texas, USA). The coding sequence for the murine *LOX-1* tagged with FLAG sequence and fused with human growth hormone polyA signal by PCR amplification was inserted into *Not*I restriction site of pSP14/15 between Tie2 promoter and enhancer. The following primers were used to generate this fusion coding sequence: sense 5'-TAA**GATATC**GAGGTCCTCGACTGTTTCAG-3' (contains *EcoR*V restriction site on its 5'-end (bold underlined), and the sequence corresponding to murine *LOX-1* cDNA (bases 12-31 of *OLR1* cDNA; NCBI no. NM 138648)) and antisense 5'-GCC**AAGCTT**TCA*CTTGTCATCGTCGTCCTTGTAGTC*AATTTGCAAATGATTTGTCTTCTTC-3' (contains *Hind*III restriction site on its 5'-end (bold typed and underlined), the sequence for FLAG-tag (italic), and the sequence that corresponds to murine *LOX-1* cDNA (underlined), bases 1123-1147 of *OLR1* cDNA; NCBI no. NM 138648). The resultant targeting vector was linearized and microinjected into

fertilized eggs prepared from superovulated C57BL/6 mice. The eggs were transferred into the oviducts of pseudopregnant foster mothers. Founder mice were identified by PCR analysis using *Tie2/LOX-1* specific pair of primers: sense 5'-CCCTGCTGATACCAAGTGCC-3' (corresponds to sequence from murine Tie2 promoter region: +111 - +130 from the Tie2 transcription start) and antisense 5'-CTTCTTTGATTCCTGTGAAGCG-3' (bases 334-355 of *OLR1* cDNA; NCBI no. NM 138648). The presence of the transgene was further confirmed in founder mice by Southern blot hybridization using [^{32}P] dCTP-labeled *LOX-1*-specific probe (Figure 1B). Several lines of *LOX-1* transgene in the C57BL/6 background were established, which carry different numbers of *LOX-1* copies. To obtain *LOX-1 TG/ApoE*$^{-/-}$ double mutant mice hemizygous *LOX-1* transgenic mice, from the line carrying maximal number of copies of the transgene, were crossbred with homozygous *ApoE*$^{-/-}$ mice on a C57BL/6 background. Offspring mice, carrying *LOX-1* transgene and being heterozygous for the *ApoE* locus, were further cross-bred with homozygous *ApoE*$^{-/-}$ mice to generate *LOX-1 TG/ApoE*$^{-/-}$ double mutant mice. Animals were maintained on a standard western chow diet until they were put on a high cholesterol diet (HCD, 1.25% total cholesterol; Research Diets). They had free access to food and water and were kept at a 12 hours light/dark cycle. The homozygous mutation in the *ApoE* locus was proved by PCR analysis with the *ApoE*-specific primers as described.

RT PCR analysis

Total RNA was extracted from aortic arches using TRIzol Reagent (Invitrogen) according to the manufacturer's recommendations. Conversion of the total cellular RNA to cDNA was carried out with murine leukemia virus reverse transcriptase and random hexamers (Amersham Bioscience) in a final volume of 33 µl, using 4 µg of cDNA. All RT PCR experiments were performed using the SYBR Green JumpStart kit (Sigma).

Tissue harvesting and processing

After puncturing the left ventricle and cutting the right atrium, vessels were rinsed briefly with normal saline and the aorta was excised after removing adventitial tissue and fat. For *en face* analyses, descending aortas were opened longitudinally. For histological examination, the aortic root was embedded in OCT (optimal cutting temperature) compound (Tissue-Tek; Sakura), frozen on dry ice, and stored at –80°C. For biochemical analyses, aortic arches were shock-frozen in liquid nitrogen and stored at -80°C.

Plaque quantification

For quantification of atherosclerosis in *LOX-1 TG* and corresponding C57BL/6 wild type mice, the aortic root was embedded in OCT, Tissue-Tek; Sakura) and frozen in isopentane. Serial cross sections of the aortic root (8 µm thickness) were cut and thaw-mounted on glass slides for immunohistochemistry Oil red-O staining.

To quantify atherosclerotic plaques in *LOX-1 TG/ApoE*$^{-/-}$ double mutant mice and *ApoE*$^{-/-}$, en face analysis of the descending aorta was performed. Plaque area was visualized by fat staining (Oil red-O) and quantified (Analysis 5; SoftImaging System).

Immunohistochemistry

Cross sections of aortas were fixed with acetone, and immunostaining was performed using the following primary antibodies: rat anti-mouse CD68 (Serotec), monoclonal rat anti-mouse CD106 (VCAM-1, Serotec), monoclonal rat anti-mouse P-sel (BD Pharmingen), and monoclonal rat anti-mouse CD3 (Serotec). For immunohistochemical detection an alkaline phosphatase–labeled donkey anti-goat antibody was used. Alkaline phosphatase was visualized using naphthol AS-BI phosphate and new fuchsin as substrate. Endogenous alkaline phosphatase was blocked by levamisole. Color reactions were performed at room temperature for 15 min with reagents from Sigma. Sections were counterstained with hematoxylin, and cover slips were mounted with glycerol and gelatin. Negative controls included omission of the first antibodies and preincubation of the first antibodies with immunogenic peptides. Slides were mounted with Glycergel mounting medium (Dako) and quantified (Analysis 5; SoftImaging System).

Western blotting

Murine aortic arches were homogenized in a lysis buffer containing 50 mM Tris, pH 7.5; 1 mM EDTA, pH 8.0; 150 mM NaCl; 1 mM PMSF, and 1 mM DTT.

Total protein extracts were cleared by centrifugation and 40 µg of proteins were separated by gel electrophoresis on 10% SDS-polyacrylamide gel followed by the semi-dry transfer onto the PVDF membrane. Finally, the membrane was incubated with primary goat anti-LOX-1 antibody (R&D Systems). Primary anti-GAPDH or anti-α-tubulin (Sigma) antibodies were used as loading controls. Secondary peroxidase-labelled anti-species-specific IgG antibodies (Amersham Biosciences, GE Healthcare Europe GmbH) were diluted 1:2500 and incubated with the membranes for 1 h at room temperature. Proteins were visualized after incubation of the membranes with Amersham™ ECL Plus Western Blotting Detection Solution (Amersham Biosciences, GE Healthcare Europe GmbH) for 5 min at RT. Protein expression was quantified using Scion Image™ software and expressed as ratio to corresponding loading control.

Plasma lipids

Plasma cholesterol level was determined using Infinity™ Cholesterol (Thermo Electron Corporation Standard) and MC Cal (Abbott). Plasma triglycerides level was measured using Infinity™ Triglycerides (Thermo Electron Corporation Standard) and MC Cal (Abbott). The distribution of lipids within the plasma lipoprotein fractions was assessed by fast-performance liquid chromatography (FPLC) gel filtration using a Superose 6 HR 10/30 column (Pharmacia).

Endothelium-dependent relaxation of intact aorta

The thoracic aortas of *ApoE*$^{-/-}$ and *LOX-1 TG/ApoE*$^{-/-}$ mice were isolated

after 20 weeks of HCD. Aortas were dissected free, excised and placed into cold modified Krebs-Ringer solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.18 and calcium disodium EDTA 0.026, glucose 11.1 (control solution). The blood vessels were cut into rings (2 mm in length). The rings were suspended in organ chambers containing control solution (37°C) aerated with 95% O_2 and 5% of CO_2. They were connected to a force transductor (Powerlab Model ML785and ML119). Changes in isometric tension were recorded. The rings were stretched progressively to their optimal resting tension (0.75 g) and were allowed to equilibrate for 90 minutes. Concentration-response curves were obtained in a cumulative way. To study endothelium-depended relaxations to acetylcholine, the preparations were exposed to U 46619 [in order to obtain 50-70% of response to KCl (60 mM)]. Sodium nitroprusside (SNP) was applied to study the endothelium-independent relaxation.

Isolation of murine endothelial cells

Murine aortic ECs were isolated as described previously [31]. Briefly, 8 week old male mice were sacrificed by cervical dislocation. Animals were perfused via left ventricle puncture with 5ml of normal saline, followed by collagenase type II (580U/ml, dissolved in serum free DMEM, Worthington). After ligation with a silk thread, the thoracic aorta was removed and incubated for 45 minutes in 37^{o}C. ECs were flushed with 5ml of DMEM containing 20% FBS and plated on 0.1% gelatin-covered plates.

Lipid uptake

EC or macrophages were stimulated with 10ug/ml Dil-oxLDL (Intracell). After 6 hours cells have been harvested and stained with anti-mouse CD105 antibody (Pharmingen), followed by incubation with FITC-labeled anti-rat secondary antibody (Molecular Probes, cat no: A-11006). Mean fluorescence of CD105-gated cells was monitored with FACS (BD, Canto II) and analysis of the data was performed using FlowJo software.

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Legends to Figures and Tables

Figure 1. Generation and characterization of endothelial-specific *LOX-1 TG* mice. (A) Scheme of endothelial-specific *LOX-1* transgenic construct. (B) Southern blot analysis of F1 generation. Genomic DNA was digested with *Bam*HI, and hybridized with *LOX-1*-specific radioactively-labeled probe. (C) Real time PCR analysis. Total RNA isolated from either aorta (a) or kidney (k) of three different transgenic lines 5048.54, 5048.24, and 5048.1 transformed into cDNA and amplified with *LOX-1*-specific primers. (D) Western blotting of aortic lysates using anti-murine LOX-1 antibody. Recombinant truncated version of murine LOX-1 used as a positive control. (E) Aortic cross sections from C57BL/6 wild type (WT) (left) and *LOX-1 TG* (right) stained with anti-murine LOX-1 antibody. Bar = 100 μm.

Figure 2. Functionality of LOX-1 construct. (A) Representative graph of the EC culture purity based on anti-CD105 staining. (B) Murine ECs isolated from WT and *LOX-1 TG* mice incubated with DiI-oxLDL for 6 hours, stained with an anti-mouse CD105 antibody. Mean fluorescence of the CD105 positive cells monitored with FACS; n=4. $^{**}P<0.01$; unpaired two-tailed Student *t*-test. (C) Representative picture of ECs stimulated with oxLDL for 6 hours and stained with oil-red O (ORO). Values are expressed as mean ± SEM.

Figure 3. Increased aortic fatty streak formation in *LOX-1 TG* mice. (A) Cross sections through aortic roots of WT and *LOX-1 TG* mice after 30 weeks of HCD

stained with ORO and (B) corresponding quantification. *P<0.05; unpaired two-tailed Student *t*-test. Values are expressed as mean ± SEM. Bar = 100 μm.

Figure 4. Increased expression of adhesion molecules and extravasation of macrophages in *LOX-1 TG* mice. Increased expression of messenger RNA for (A) *VCAM-1* and (B) *E-sel* in *LOX-1 TG* (n=11) mice compared with WT mice (n=11, *P<0.05). Cross sections of aortic roots from WT and *LOX-1 TG* mice stained with (C) antibody against VCAM-1, and (E) antibody against CD68 with corresponding quantifications ((D) and (F) respectively); unpaired two-tailed Student *t*-test. Values are expressed as mean ± SEM. Bar = 100 μm.

Figure 5. Increased aortic plaque formation in *LOX-1 TG/ApoE$^{-/-}$* mice. (A) *En face* ORO staining of descending aortas of *ApoE$^{-/-}$* or *LOX-1 TG/ApoE$^{-/-}$* mice after 20 weeks of HCD. (B) Increased atherosclerotic burden in *LOX-1 TG/ApoE$^{-/-}$* mice compared with *ApoE$^{-/-}$* controls (n=9-12, *P<0.05) mice; unpaired two-tailed Student *t*-test. Values are expressed as mean ± SEM.

Figure 6. Enhanced VCAM-1 and P-sel expression in *LOX-1 TG/ApoE$^{-/-}$* mice. Cross sections of aortic roots from *ApoE$^{-/-}$* or *LOX-1 TG/ApoE$^{-/-}$* mice stained with (A) antibody against VCAM-1 with corresponding quantification (B; n=7; *P<0.05). (C) Increased expression of messenger RNA for *VCAM-1* in *LOX-1 TG/ApoE$^{-/-}$* mice compared with *ApoE$^{-/-}$* mice (n=13, *P<0.05). Cross sections of aortic roots from *ApoE$^{-/-}$* or *LOX-1 TG/ApoE$^{-/-}$* mice stained with (D) antibody against P-sel with

corresponding quantification (E; n=9; $^{**}P<0.01$); unpaired two-tailed Student *t*-test. Values are expressed as mean ± SEM. Bar = 100 μm.

Figure 7. Increased extravasation of macrophages and lymphocytes in *LOX-1 TG/ApoE*$^{-/-}$ mice. Cross sections of aortic roots from *ApoE*$^{-/-}$ or *LOX-1 TG/ApoE*$^{-/-}$ mice stained with (A) antibody against CD68, or (C) antibody against CD3 (arrows indicate positive staining) with corresponding quantifications ((B) and (D) respectively). $^{*}P<0.05$; unpaired two-tailed Student *t*-test. Values are expressed as mean ± SEM. Bar = 100 μm.

Table 1. Plasma lipid levels.

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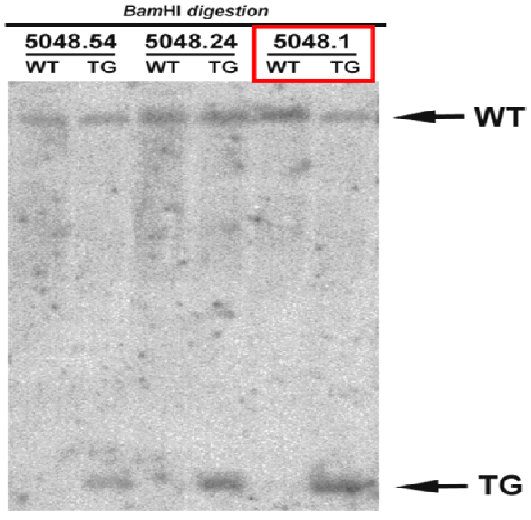
Figure 1

A

B



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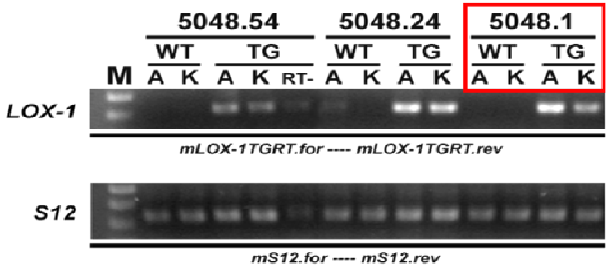

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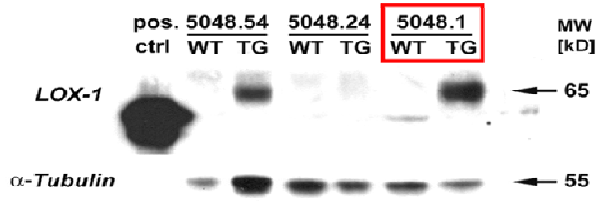

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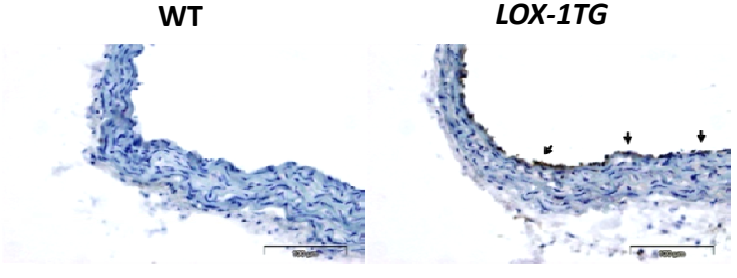

Figure 2

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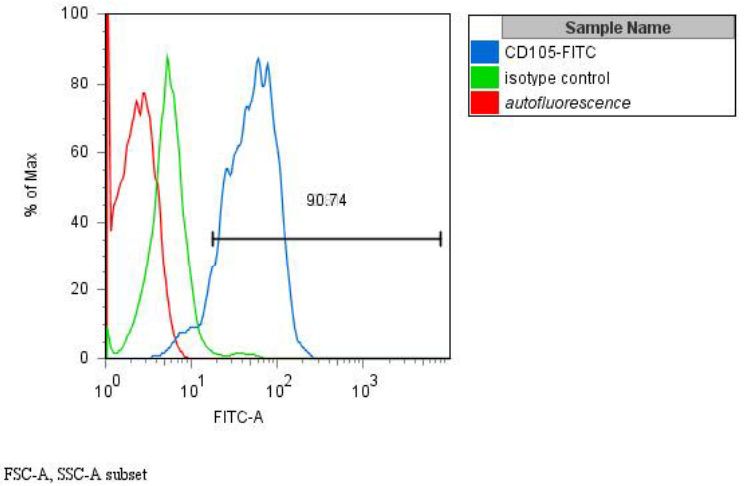
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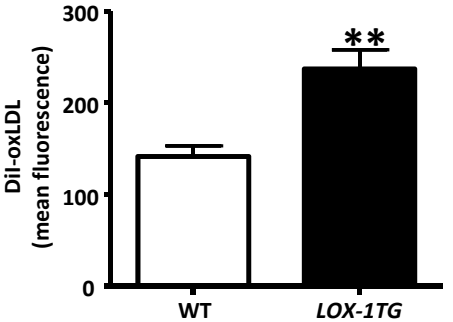
B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(mean fluorescence)
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WT
LOX-1TG

C

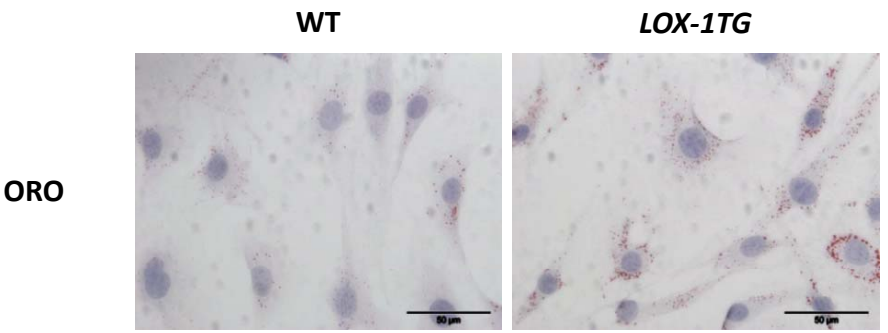
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Figure 3

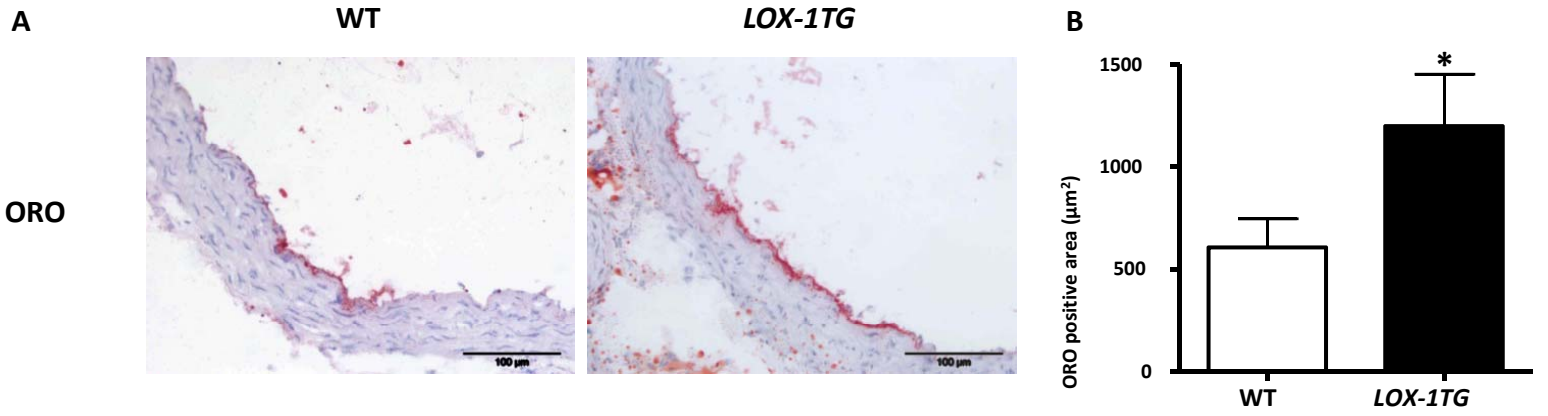
A
WT
LOX-1TG
ORO
100 µm
100 µm
B
1500
1000
500
0
ORO positive area (µm²)
*
WT
LOX-1TG

Figure 4

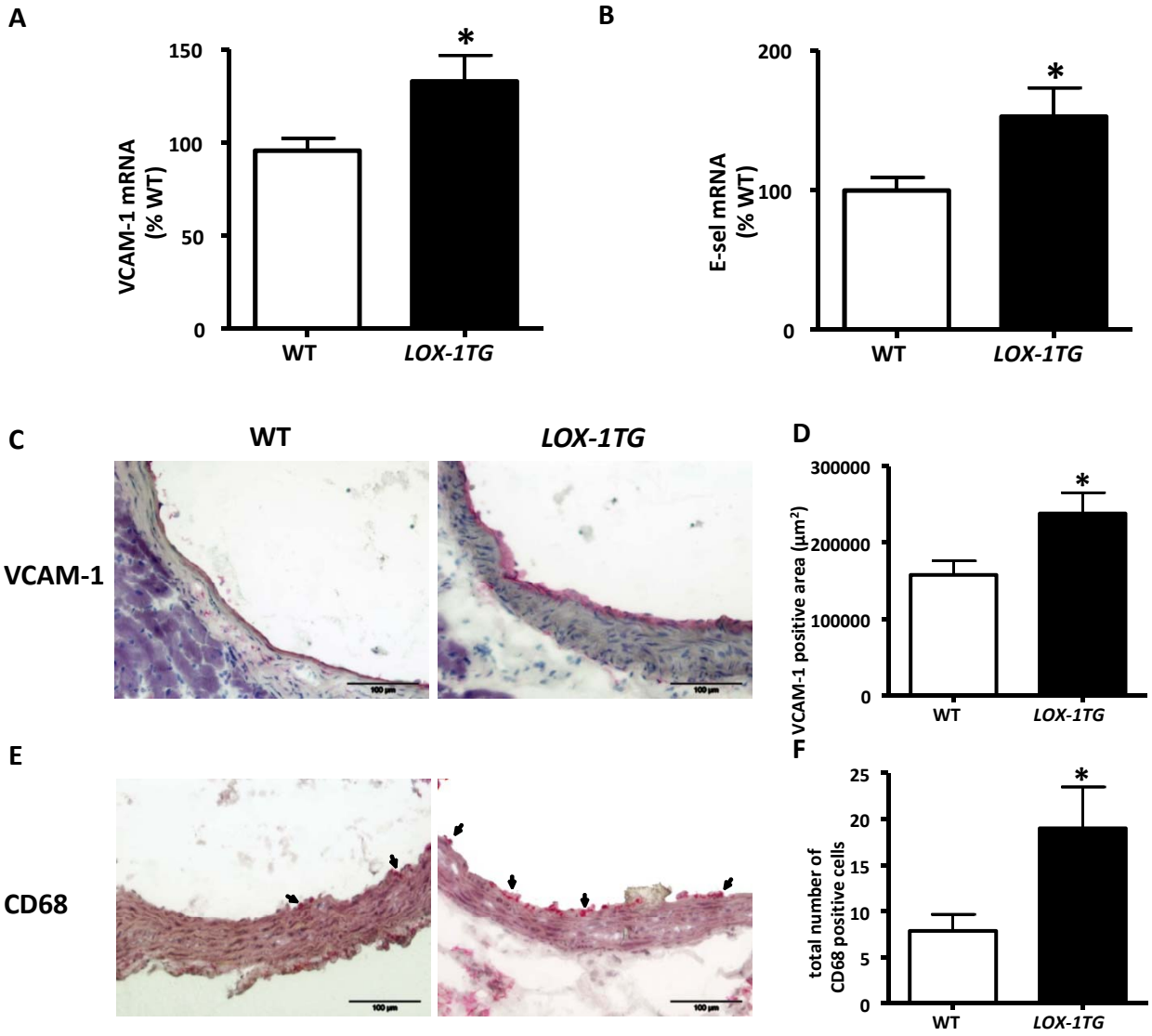
A
VCAM-1 mRNA (% WT)
150
100
50
0
*
WT
LOX-1TG
B
E-sel mRNA (% WT)
200
100
0
*
WT
LOX-1TG
C
WT
LOX-1TG
VCAM-1
D
VCAM-1 positive area (μm²)
300000
200000
100000
0
*
WT
LOX-1TG
E
CD68
F
total number of CD68 positive cells
25
20
15
10
5
0
*
WT
LOX-1TG

Figure 5

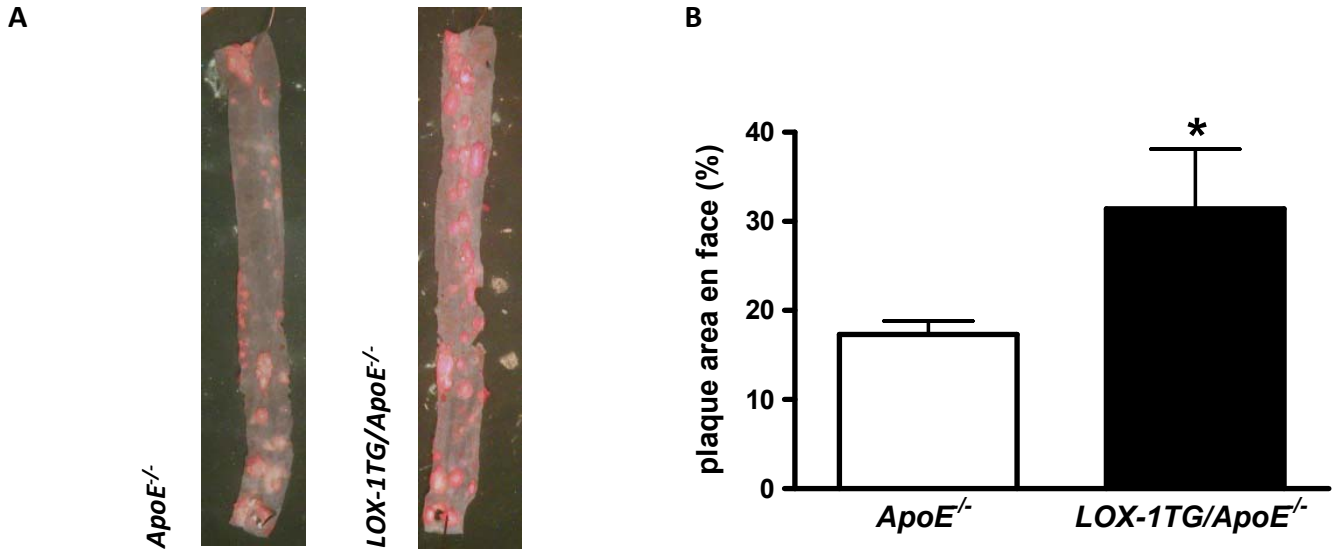
A
ApoE-/-
LOX-1TG/ApoE-/-
B
plaque area en face (%)
40
30
20
10
0
*
ApoE-/-
LOX-1TG/ApoE-/-

Figure 6

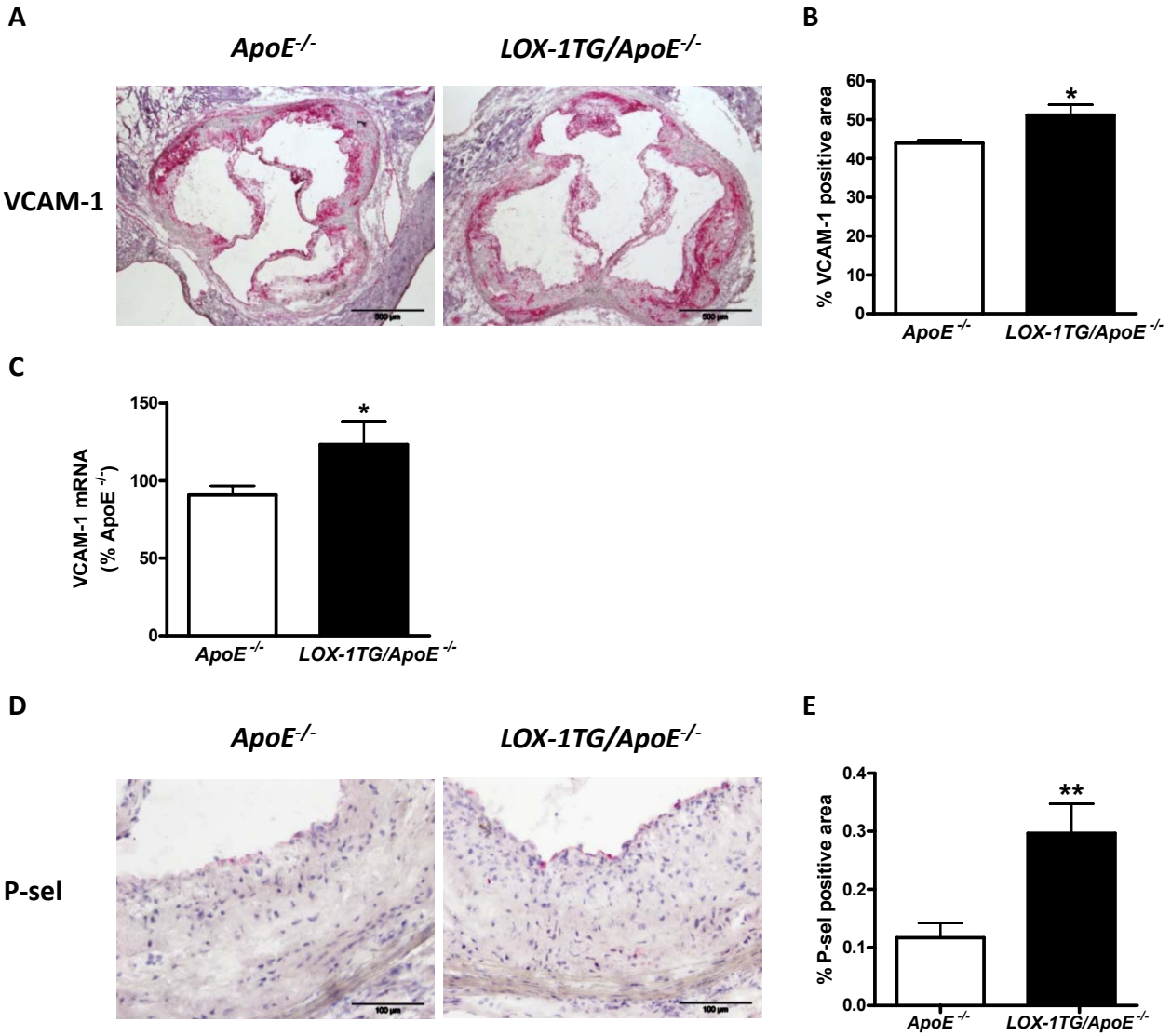
A
ApoE-/-
LOX-1TG/ApoE-/-
VCAM-1
B
% VCAM-1 positive area
60
50
40
30
20
10
0
*
ApoE -/-
LOX-1TG/ApoE -/-
C
VCAM-1 mRNA
(% ApoE -/-)
150
100
50
0
*
ApoE -/-
LOX-1TG/ApoE -/-
D
ApoE-/-
LOX-1TG/ApoE-/-
P-sel
E
% P-sel positive area
0.4
0.3
0.2
0.1
0.0
**
ApoE -/-
LOX-1TG/ApoE -/-

Figure 7

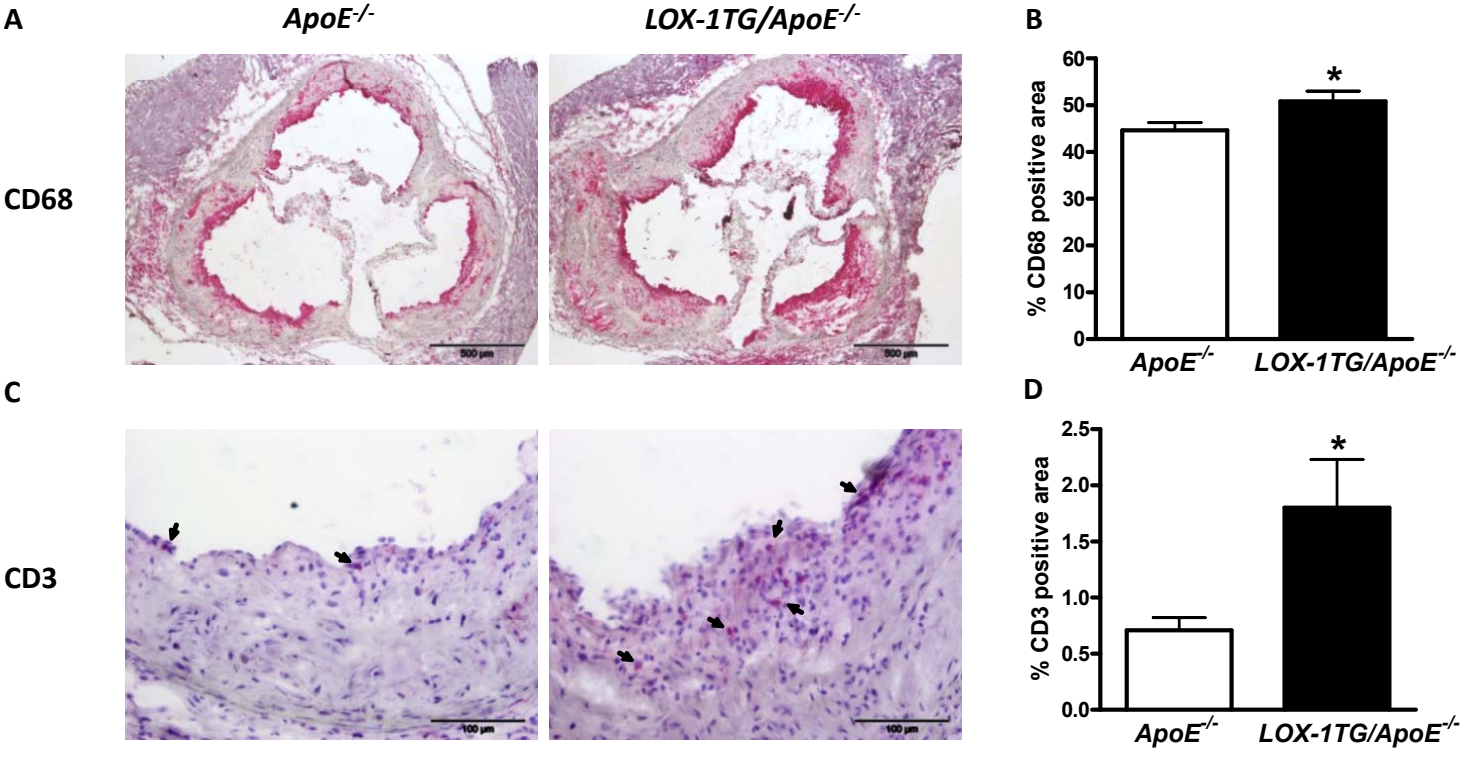
A
ApoE-/-
LOX-1TG/ApoE-/-
CD68
B
% CD68 positive area
60
50
40
30
20
10
0
*
ApoE-/-
LOX-1TG/ApoE-/-
C
CD3
D
% CD3 positive area
2.5
2.0
1.5
1.0
0.5
0.0
*
ApoE-/-
LOX-1TG/ApoE-/-

Table 1

genotype	cholesterol (mmol/L)	triglyceride (mmol/L)	free fatty acids (mmol/L)
WT	5.64 ± 1.20	0.77 ± 0.07	0.30 ± 0.06
LOX-1 TG	6.27 ± 0.94	0.75 ± 0.03	0.28 ± 0.08
LOX-1 WT/ApoE$^{-/-}$	19.97 ± 2.98	0.84 ± 0.18	0.44 ± 0.07
LOX-1 TG/ApoE$^{-/-}$	16.98 ± 2.83	1.13 ± 0.28	0.36 ± 0.06

8. Publications that did not contribute to the work

Ricci, R., Sumara, G., Sumara, I., **Rozenberg, I**., Kurrer, M., Akhmedov, A., Hersberger, M., Eriksson, U., Eberli, F.R., Becher, B., et al. 2004. Requirement of JNK2 for scavenger receptor A-mediated foam cell formation in atherogenesis. *Science* 306:1558-1561.

Matter, C.M., **Rozenberg, I**., Jaschko, A., Greutert, H., Kurz, D.J., Wnendt, S., Kuttler, B., Joch, H., Grunenfelder, J., Zund, G., et al. 2006. Effects of tacrolimus or sirolimus on proliferation of vascular smooth muscle and endothelial cells. *J Cardiovasc Pharmacol* 48:286-292.

Tanner, F.C., van der Loo, B., Shaw, S., Greutert, H., Bachschmid, M.M., Berrozpe, M., **Rozenberg, I**., Blau, N., Siebenmann, R., Schmidli, J., et al. 2007. Inactivity of nitric oxide synthase gene in the atherosclerotic human carotid artery. *Basic Res Cardiol* 102:308-317.

Payeli, S.K., Schiene-Fischer, C., Steffel, J., Camici, G.G., **Rozenberg, I**., Luscher, T.F., and Tanner, F.C. 2008. Cyclophilin A differentially activates monocytes and endothelial cells: role of purity, activity, and endotoxin contamination in commercial preparations. *Atherosclerosis* 197:564-571.

9. Discussion of the results

The aim of this thesis was to find and characterize new genes involved in the pathogenesis of atherosclerosis. We studied several molecules, which could be relevant for different aspects of disease development, namely: (i) histamine receptors, which are well known mediators of inflammatory response [12, 66, 67], (ii) L-sel, an adhesion molecule responsible for early interactions between leukocytes and activated endothelium [18, 28], and (iii) LOX-1, a scavenger receptor for oxLDL expressed by ECs [41]. Genetically modified mice were used to assess the importance of these molecules in atherosclerosis development.

"Histamine H1 receptor promotes atherosclerotic lesion formation by increasing vascular permeability for low density lipoproteins"

Histamine was demonstrated to regulate several processes affecting atherosclerosis development *in vitro*, such as expression of adhesion molecules, and proinflammatory cytokines, Th1/Th2 balance and vascular permeability [12, 68]. However, the relevance of histamine-mediated signalling in atherogenesis *in vivo* remained unclear. We demonstrated for the first time that both pharmacological blockade and genetic deletion of *H1R*, but not *H2R*, attenuates the development of atherosclerosis in *ApoE*$^{-/-}$ mice. Genetic deletion of *H1R* displayed a more pronounced effect on atherosclerosis development compared to pharmacological blockade (60% versus 40% of atherosclerotic plaque reduction). This may well be related to a lower efficiency of the pharmacological

inhibition compared to the genetic approach. Indeed, it is to be expected that pharmacological blockade of a receptor *in vivo* can not be complete; while genetically modified animals do not express the receptor at all.

Increased vascular permeability is an important consequence of ECs activation and leads to LDL accumulation in the arterial intima, favouring the formation of atherosclerotic plaques [1, 69]. Acute stimulation with histamine is known to increase vascular permeability *in vitro* as well as *in vivo*, particularly in the context of acute allergic reactions [70]. Indeed, treatment with H1R antagonists is a highly effective therapy against allergic diseases [71-74]. Since expression of HDC increases during atherosclerosis development, atherosclerotic plaques, but not normal arteries, contain large amounts of histamine [11]. The enhanced histamine concentration in the vascular wall may indeed affect vascular permeability chronically during atherosclerosis development. To address this issue, we monitored aortic permeability of *ApoE*$^{-/-}$ *H1R*$^{-/-}$ mice to radiolabeled LDL. Lack of *H1R* clearly attenuated vascular permeability in *ApoE*$^{-/-}$ animals. This effect was observed in animals without macroscopically visible atherosclerotic plaques as well as in mice with more advanced atherosclerosis. Indeed, neither the occurrence nor the magnitude of this effect depended on the stage of atherosclerosis; suggesting a primary difference. Consistent with this observation, the total fat content in atherosclerotic plaque was reduced upon *H1R* deletion as compared to controls. These data indicate that histamine acting via H1R is a critical determinant of vascular permeability during atherosclerosis development. In line with this interpretation,

bone marrow transplantation revealed that the presence of H1R on vascular cells, but not on BM-derived cells, promotes atherogenesis.

ApoE$^{-/-}$ *H1R*$^{-/-}$ animals exhibited reduced numbers of both, macrophages and Th cells in atherosclerotic plaques compared to *ApoE*$^{-/-}$ mice. This was associated with decreased abundance of CCL5 in aorta of *ApoE*$^{-/-}$ *H1R*$^{-/-}$ mice. Moreover, atherosclerotic, but not normal animals, lacking the *H1R* exhibited reduced spleen weight and blood lymphocytes number. We can speculate that reduced local and systemic inflammation in *ApoE*$^{-/-}$ *H1R*$^{-/-}$ mice is a consequence of diminished aortic permeability. Indeed, it has been demonstrated that histamine-mediated permeability is associated with increased leukocyte accumulation [75]. Moreover, mmLDL may act like chemoattractant [1]; thus, impaired accumulation of LDL could account for reduced leukocyte extravasation.

To evaluate the contribution of vascular versus BM-derived cells in atherosclerosis development in *ApoE*$^{-/-}$ *H1R*$^{-/-}$ mice, BM transplantation was performed. This experiment revealed that the presence of *H1R* on vascular, but not bone marrow-derived cells, promotes atherosclerotic plaque formation. These data support the interpretation that differences in inflammation in animals lacking *H1R* are secondary to reduced vascular permeability in these animals.

In conclusion, this study demonstrates that *H1R,* but not *H2R,* is crucially involved in atherosclerosis, most likely due to its role as a mediator of vascular permeability. However, we did not discriminate between vascular permeability and LDL retention in the vessel wall, which could be an alternative explanation of

decreased vascular LDL abundance in animals lacking *H1R*. A long-term *in-vivo* permeability assay as well as studies demonstrating the exact location of the LDL within atherosclerotic plaque of animals lacking the *H1R* is required to address these questions.

"Accelerated early atherosclerosis in mice deficient in *L-selectin*"

Atherosclerosis is a result of the chronic inflammation induced by accumulation of cholesterol and leukocytes into the arterial intima [1]. L-sel belongs to selectin family of adhesion molecules regulating initial stages of leukocytes extravasation [18, 28]. Therefore, we hypothesize that L-sel deletion reduces atherosclerosis development due to reduction in leukocyte migration into atherosclerotic plaques. To test this hypothesis we generated *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ and compared them to *ApoE*$^{-/-}$ controls during early (no HCD, 6 weeks of HCD) and more advanced (12 weeks of a HCD) stages of the disease. Surprisingly, we found that deletion of *L-sel* accelerates early, but not advanced atherosclerosis.

We used intravital microscopy to monitor whether deletion of *L-sel* affects leukocyte capture and rolling during atherosclerosis development. We did not, however, observe any differences between *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ animals and corresponding *ApoE*$^{-/-}$ controls. These results are in line with some previous reports which describe overlapping function for all selectin family members [40, 76]. Moreover, most of the studies describing the importance of L-sel in leukocyte rolling along activated endothelium focused on venules and arterioles [30, 77], whereas atherosclerosis is a disease of large arteries. Finally, some studies were

performed under different experimental conditions, for example after adaptive transfer, which may affect the activatory state and intrinsic properties of transferred leukocytes [39]. In line with this finding, we did not detect any difference in cellular composition of atherosclerotic plaques. Indeed, plaque morphology, number of macrophages and T-cells accumulated into plaques were similar in *ApoE*$^{-/-}$ *L-sel*$^{-/-}$ and *ApoE*$^{-/-}$ mice.

As described previously, *L-sel* deficient mice exhibited altered leukocyte distribution due to defective lymphocyte homing to peripheral lymph nodes [28]. Indeed, we observed significantly decreased size and cellularity of lymph nodes, increased numbers of blood lymphocytes, and compensatory increase in spleen size. This alteration occurred independently from the stage of atherosclerosis development, thus it cannot explain the difference in atherosclerosis development.

In summary, this study demonstrates the complexity of the immune response during atherosclerosis development. We showed that *L-sel* deletion results in leukocyte redistribution and acceleration of early, but not advanced atherosclerosis. We suggest that *L-sel* deletion results in very complex changes in the immune system affecting atherosclerosis. The mechanism of this phenomenon, however, remains unclear and requires further investigation.

"Endothelial overexpression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) increases aortic LDL uptake and plaque formation *in vivo*"

One of the important mechanisms of ECs activation and dysfunction is associated with the uptake of modified LDL particles [13, 44]. ECs activation precedes formation of atherosclerotic changes and is an important event in early atherogenesis [78]. While macrophages migrated into the vessel wall scavenge oxLDL using SR-A and CD36, ECs take up oxLDL mainly via LOX-1 [13, 41, 49]. Indeed, it has been demonstrated *in vitro* that oxLDL uptake initiates a cascade of molecular events leading to ECs activation [13].

We have generated transgenic animals overexpressing LOX-1 specifically in the ECs. We studied these animals in the context of early atherosclerosis after 30 weeks of HCD. In addition, we crossed bred these mice with *ApoE*$^{-/-}$ animals and fed them for 20 weeks a HCD to monitor the relevance of LOX-1 in advanced atherosclerosis. Recently, it has been demonstrated that animals lacking *LOX-1* exhibit decreased atherosclerotic burden [57]. In line with this finding, LOX-1 overexpression accelerated both, early and advanced atherosclerosis, which was associated with impaired endothelium-dependent relaxation to acetylcholine.

It was demonstrated that oxLDL uptake by ECs results in enhanced adhesion molecules expression [13]. Indeed we observed increased expression of VCAM-1 and E-sel in *LOX-1 TG* mice as well as VCAM-1 and P-sel in *LOX-1* TG/*ApoE*$^{-/-}$ mice as compared to corresponding controls. The discrepancy

between *LOX-1 TG* and *LOX-1* TG/*ApoE*$^{-/-}$ mice in the expression pattern of adhesion molecules is not surprising, considering the different stage of disease development in these animals [79]. In line with this, we found increased accumulation of macrophages in atherosclerotic plaques of both *LOX-1 TG* and *LOX-1* TG/*ApoE*$^{-/-}$ mice.

In conclusion, in this study we showed that endothelial overexpression of LOX-1 is associated with increased atherosclerosis development due to enhanced endothelial dysfunction. However, we did not dissect the molecular pathways connecting LOX-1 overexpression with endothelial dysfunction. It was demonstrated *in vitro* that NFκB is involved in oxLDL-mediated ECs activation; however, additional studies are required to prove this observation *in vivo*.

We believe that these novel observations improve the current understanding of the pathogenesis of atherosclerosis. In addition, they have potential therapeutic implications, since (i) histamine receptor blockers are widely used to prevent or treat the acute increase in vascular permeability during allergic reactions. Furthermore, (ii) development of strategies targeting early events in atherogenesis, such as endothelial activation or leukocyte capture, may open new therapeutic approaches for the prevention as well as for the treatment of atherosclerosis.

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